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STUDIES ON SOME QUATERNARY COMPOUNDS WITH AN INHIBITORY EFFECT ON THE GASTRIC SECRETION OF RATS

BY

STEEN ANTONSEN

MUNKSGAARD COPENHAGEN 1968

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FROM THE PHARMACOLOGICAL LABORATORY,
ALFRED BENZON LTD
29 HALMTORVET, DK 1700 COPENHAGEN V

Studies on some Quaternary Compounds with an Inhibitory Effect on the Gastric Secretion of Rats

By

STEEN ANTONSEN

Department of Pharmacology
A/S ALFRED BENZON
Copenhagen, Denmark, 1968

To

Dr. Boje Benzon

whose spirit of freedom
has made this work possible

Denne afhandling er sammen med de nedennævnte seks tidligere publicerede afhandlinger af Danmarks farmaceutiske Højskole antaget til offentligt at forsvares for den farmaceutiske doktorgrad

København, den 29 maj 1968

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Antonsen, S Some quaternary, non-anticholinergic compounds with an inhibitory action on "Shay-rats" *Acta pharmacol et toxicol* 1965a, 23, 154–164

Antonsen, S Gastric secretion of rats after test meals *Acta pharmacol et toxicol* 1965b, 23, 165–178

Antonsen, S Application of test meal method for investigating the effect of some quaternary non-anticholinergic substances on the rat stomach *Acta pharmacol et toxicol* 1965c, 23, 179–188

Antonsen, S Comparison of the inhibitory effects of tertiary and quaternary compounds on the gastric secretion of rats *Acta pharmacol et toxicol* 1967a, 25, 405–419

Antonsen, S Comparison of the stimulating effects of gastrin and histamine on the gastric secretion of rats *Acta pharmacol et toxicol* 1967b, 25, 420–426

Antonsen, S Antagonism of gastrin- and histamine-stimulated gastric secretion in rats *Acta pharmacol et toxicol* 1967c, 25, 427–434

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Preface

The present work has been carried out during my long employment at the Pharmacological Laboratory, ALFRED BENZON Ltd

I am greatly indebted to the management of the firm for the excellent working conditions offered me to carry through my investigations

My sincere thanks are due specially to Mr Erik Hermann, Head of the Development and Research Department of the firm, for his valuable encouragement and aid

Further, I wish to thank Mrs Gyda Hegermann Nielsen and Mr Sune Jespersen, my colleagues at the Pharmacological Laboratory, for their helpfulness and interest in my studies Miss I Hundborg, Miss H S Eckardt and Mr B J Andersen have yielded skilled technical aid, for which I thank them

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However, contrary to expectation, the substances prepared were found to have an independent inhibitory effect on the gastric secretion in our test methods employed on rats If the test methods were representative of the clinical effect, there was a chance that the substances might be of therapeutic value, without having the side effects of anticholinergic drugs

The first investigations (ANTONSEN 1965a) showed that in pyloric-ligated rats (Shay tests) the quaternary, non anticholinergic compounds inhibit the secretion of gastric juice and protect against ulceration These actions are not explainable on the basis of the other pharmacological properties of the drugs

A review of the literature revelated no explanation of the observed actions

A test meal method (ANTONSEN 1965b) was developed to obtain further information on the effects of the test substances on rat stomachs Using this method a histamine-stimulated secretion was inhibited by anticholinergic compounds, but not by antihistaminic

In these experiments the test substances had a considerable inhibitory effect on the gastric secretion and caused retention of the test meal in the stomach (ANTONSEN 1965c) The effect was observed both after parenteral and after oral administration, and the duration of the effect could be measured

To clarify the significance of the quaternary structure the test substances were compared with their tertiary analogues (ANTONSEN 1967a) A quaternary,

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anticholinergic compound and its tertiary analogue were also included Shay-tests and test meals were employed The quaternary compounds had a stronger effect than the tertiary A quantitative comparison is difficult, however, the results depending on conditions of absorption and duration of effect As in previous assays, an increased mydriatic effect was demonstrable of a quaternary, anticholinergic compound, when this was administered together with one of the quaternary, non-anticholinergic drugs

Next, (ANTONSEN 1967b) a comparison was drawn between the stimulating effects of a gastrin preparation and histamine in test meal experiments and on Shay rats The Shay-test comprised both two- and four hour pyloric-ligation periods as well as experiments with and without ligation of the œsophagus *The Shay-test was less suitable than the test meal method for* quantitative measurement of the gastrin-stimulated secretion Further, the test meal method served to disclose a difference between histamine and gastrin with regard to the mode of action on gastric emptying

Test meal experiments were conducted to compare the influences of antihistaminic and anticholinergic drugs on gastrin- and histamine-stimulated secretion (ANTONSEN 1967c) Analogous investigations of the quaternary, non-anticholinergic compounds showed these to be more active against the gastrin-stimulated secretion than against histamine-stimulation While U247–51, which has a certain sympathomimetic action, always displayed a weaker effect than U247–73 on spontaneous and histamine-stimulated secretion, the two substances were equally active against gastrin-stimulated secretion

By the test methods employed the new quaternary compounds thus had a distinct inhibitory effect on both the spontaneous and the stimulated gastric *secretion of the rat, without possessing at the same time the pharmacological* actions normally explaining this effect

U247–51 and U247–73 are alike chemically, but their pharmacological actions differ in several respects The inhibitory effect of the two substances on the stomach must be due to a mechanism of action *demonstrable in both*

Research on the properties of the substances was continued to find this mechanism of action and, specially, to investigate how much importance may be attached to results of experiments on rat stomachs

Even though the rat is the animal most frequently used for testing the effects of drugs on the stomach, it is in many respects unfit for this purpose The rat being a rodent, its digestive apparatus differs appreciably from the human It is more resistant to histamine than those of humans and of other animal species (KOMAROV *et al* 1941, ROSE & BROWNE 1938, KYLE & WILBOURN 1956, KAHLSON *et al* 1961a, GROSSMAN 1967, THOMPSON & LEE 1967) The sex hormones have an influence on the ulceration, but this *depends on the test method* (ANTONSEN 1955, KAHLSON *et al* 1964b, WILSON

1966) CODE (1965) has commented that "The rat is an odd beast, and predictions are likely to be misleading"

Rats are used extensively as experimental animals because they are easily available in large relatively uniform groups Further it was realized fairly early that ulcers develop in the gastric wall of the rat after ligation of the stomach for a few hours (SHAY *et al* 1945)

This test gained considerable ground for screening of new substances after it had been found that development of ulcers were prevented by the drugs commonly used in ulcer therapy (ANTONSEN 1965a)

The present work gives an account of some pharmacological studies carried out to clarify, if possible, which mechanisms may be responsible for the inhibitory effects of the new test substances on the stomach Further, some supplementary investigations on the stomach of the rat are included

The stomach being ligated during the experimental period, a normal absorption of orally administered test substance during this period is impossible Accordingly parenteral administration is often employed This cannot, however, give a correct picture of the effects of such substances as are meant to be given by mouth We therefore conducted a series of experiments with a view to finding a method by which to introduce the test substances into the ligated part of the alimentary tract

In addition to the Shay-test, we employed a test meal method on rats (ANTONSEN 1965b) and also studied stress induced ulceration Finally, we employed a special method for measuring the gastric secretion in anaesthetized rats (ANTONSEN 1959)

Histamine was generally employed for stimulation of gastric secretion, but a gastrin preparation was used for a few comparative tests

anticholinergic compound and its tertiary analogue were also included. Shay-tests and test meals were employed. The quaternary compounds had a stronger effect than the tertiary. A quantitative comparison is difficult, however, the results depending on conditions of absorption and duration of effect. As in previous assays, an increased mydriatic effect was demonstrable of a quaternary, anticholinergic compound, when this was administered together with one of the quaternary, non-anticholinergic drugs.

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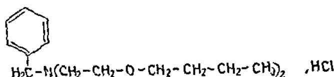
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The tertiary analogue to U243-73 is *U247-90*

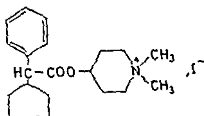
N,N-di-(2 Butoxyethyl)-benzylamine, HCl



Mol 344 0 Solubility > 100 mg/ml M p 92°C

The new, quaternary, anticholinergic compound is

U247-96 1-Methyl-4-piperidyl cyclopentyl-phenylacetate methoiodide

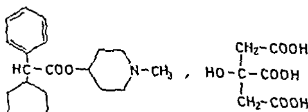


Mol 443 4 Solubility 25 mg/ml M p 148–150°C

The compound was not tested clinically, but its spasmolytic properties have been described previously (ANTONSEN 1967a)

The tertiary compound corresponding to U247-96, whose spasmolytic effects have also been described (ANTONSEN 1967a) is

U247-95 1-Methyl-4 piperidyl cyclopentyl phenylacetate citrate



Mol 493 6 Solubility 16 mg ml M p 140–143°C.

As in previous studies (ANTONSEN 1953, ANTONSEN & *NIELSEN 1963*, ANTONSEN 1965a,b,c, 1967a,c), comparisons were, finally, drawn with the quaternary, anticholinergic compound Metropin®, whose clinical effect is known

Chapter 1

Chemical Configurations of the Tested Compounds

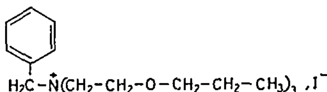
As stated in the introduction, the poor absorption of the compounds from the gastro-intestinal canal is due to their quaternary structure

To clarify the significance of this, comparisons with the corresponding tertiary compounds were included in some of the investigations

Comparisons were, further, drawn with a new quaternary, anticholinergic compound and its tertiary analogue, and a quaternary, anticholinergic compound of a known clinical effect

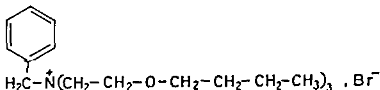
The quaternary, non-anticholinergic compounds are

U247–51 N,N,N,-tri-(2-Propoxyethyl)-benzylammonium iodide



Mol 493 5 Solubility 5 mg/ml H_2O M p 102°C

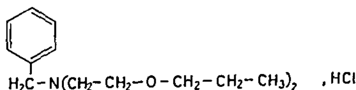
U247–73 N,N,N,-tri-(2-Butoxyethyl) benzylammonium bromide



Mol 488 6 Solubility 7 mg/ml H_2O M p 106°C

The tertiary analogue to U247–51 is *U247–125*

N,N,-di (2-Propoxyethyl)-benzylamine, HCl

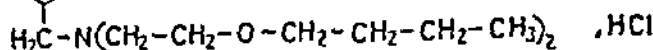


Mol 315 9 Solubility > 100 mg/ml H_2O M p 106°C

The tertiary analogue to U243-73 is *U247-90*

N,N-di (2 Butoxyethyl) benzylamine, HCl

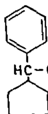


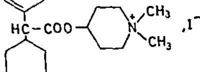


Mol 344.0 Solubility >100 mg/ml M p 92°C

The new, quaternary, anticholinergic compound is

U247-96 1-Methyl-4 piperidyl cyclopentyl phenylacetate methoiodide



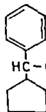


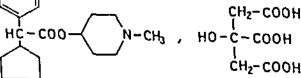
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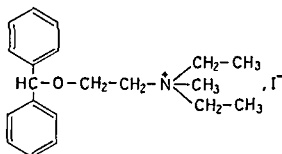




Mol 493.6 Solubility 16 mg ml M p 140–143°C

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Metropin® 2-(Diphenylmethoxy)-N,N-diethyl-N-methyl ethylammonium iodide



Mol 425 3 Solubility 3 mg/ml M p 158–160°C

Metropin® is the Danish trade name, thus being not identical with methylatropine nitrate, which in other countries is registered under this name

Chapter 2

Pharmacological Studies of the Quaternary, Non-Anticholinergic Compounds

1 *Acute Toxicity of the Quaternary and the Tertiary Compounds*

The acute toxicity of the quaternary compounds was compared with that of the tertiary in experiments on female mice

The oral doses were given with stomach tube in the form of aqueous solutions to fasting mice Parenterally the substances were given in 0 9% NaCl

LD_{50} was found for the compounds after intravenous, intraperitoneal, and oral administration Further, LD_{100} i v was measured by continuous intravenous infusion according to the method indicated by HINT & RICHTER (1958) The substances were given in concentrations which killed the animals within 1½ to 2 minutes

The results are shown in *table 1*

Table 1

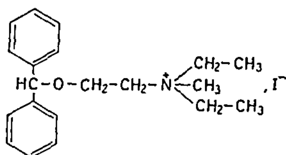
Acute toxicity of the test substances when given to female mice LD_{50} ± standard error calculated according to MILLER & TAINTER (1944) LD_{100} indicated as mean value ± s e m The bracketed figures represent the numbers of mice used for the determination

	LD_{50} orally	LD_{50} i p	LD_{50} i v	LD_{100} i v
U247-51	153 ± 5(36)	68 ± 5(19)	40 ± 2(26)	103 ± 2(10)
U247-125	830 ± 18(47)	295 ± 16(36)	27 ± 2(28)	106 ± 4(10)
U247-73	195 ± 18(46)	25 ± 3(20)	27 ± 4(19)	139 ± 11(10)
U247-90	1075 ± 53(18)	352 ± 25(24)	44 ± 2(20)	182 ± 20(10)

After oral doses large enough to kill the animals *these died within one hour*, in agreement with the fact that a rapid, but incomplete absorption takes place from the gastro-intestinal canal (LEVINE 1966)

Intraperitoneal injection provoked excitation proceeding in some instances to convulsions Then followed sedation and loss of *rising reflex* until the animals died Most of the animals had *excessive diarrhoea* Intravenous injection most often had a sedative effect only

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During intravenous infusion there was first a period of unrest and excitation Then followed a period of sedation and loss of rising reflex until the animals

died The excitation was more pronounced in response to U247–51 than to U247–73, but the two quaternary compounds both caused greater excitation than the tertiary

As seen in the table, the four substances were approximately equally toxic when given intravenously After intraperitoneal and oral administration, on the other hand, the quaternary compounds were seen to be much more toxic than the tertiary In other words, the tertiary compounds were not in this way found to be absorbed to a greater extent than the quaternary from the gastro-intestinal canal

Subcutaneous injections caused local irritation and in some cases produced necroses at the site of injection on prolonged treatment In tests on rabbits given trypan blue intravenously (HOPPE *et al* 1950) the quaternary compound U247–51 and the tertiary U247–125 had approximately equal locally irritating effects

2 *Chronic Toxicity of U247–51 and U247–73*

Rats were given 10–25 or 50 mg/kg orally once daily for two months

Mice were given 5 and 10 mg/kg i p and 25 and 50 mg/kg orally once daily through 3 months

All the treated animals thrived normally, and histological examination of the animals' organs revealed no pathological conditions

3 *Tests on Humans*

Three normal subjects received daily doses of 25, 50, and 75 mg U247–51 respectively through a period of 10 weeks

Haemoglobin, leucocyte count, thrombocyte count, and G P -transaminase activity were recorded initially and again after 3, 7, and 10 weeks of medication

No significant alterations were observed during the test period, and the subjects reported no discomfort

A double-blind trial on ambulatory ulcer patients showed such favourable effects of both test substance and placebo that no significant difference could be noticed

4 *Tests on Isolated Organs*

As previously stated (ANTONSEN 1965a, 1967a), neither the quaternary nor the tertiary compounds were found to have any effects worth mentioning on the isolated *guinea pig intestine* Neither were their effects on the isolated *rabbit intestine* of any interest

In the isolated *rectus muscle* of the frog, on the other hand, antagonism to acetylcholine was demonstrated

All the test substances were compared with tubocurarine (Tubocuran®) in an organ bath of 45 ml

Administration of a suitable submaximum dose of acetylcholine was preceded by rising doses of tubocurarine and test substance alternately The dose reducing the effect of acetylcholine by half was calculated from the dose-response curves

The following mean values were found

Tubocurarine chloride	ED_{50}	60 µg	
Metropin®	ED_{50}	90 µg	(12 tests)
U247-51	ED_{50}	100 µg	(7 tests)
U247-125	ED_{50}	600 µg	(3 tests)

Neither U247-73 nor the corresponding tertiary compound U247-90 had any antagonistic effect Thus in the cases of these substances the quaternary structure is not alone responsible for the effect

For the quaternary, anticholinergic compound U247-96 ED50 was 43 µg (average of four tests), while the corresponding tertiary compound U247-95 had a weak dose nonrelevant-effect only

By adding Metropin® and U247-51 simultaneously to the bath a pure additive effect of the two substances was obtained

Addition of physostigmine to the bath during the comparison of U247-51 and tubocurarine required larger doses of both substances to obtain the same inhibitory effect, and the slopes of the dose-response curves became quite different The slope became much flatter for U247-51 than for tubocurarine, so that direct comparison was impossible In the physostigmine suspension U247-51 was nevertheless found to have a relatively weaker effect than tubocurarine, the latter having under these circumstances a four to seven times stronger effect than the former

The isolated rectus muscle preparation is unsuitable for classification of a new substance because the anticholinergic effect can be brought about by such varied substances as anticholinergic drugs, local anaesthetics, quinidine, and alkaloids (ELIO 1948, STEPHENSON 1948)

Experiments on the isolated rat diaphragm gave results showing that it is not a question of a pure curare effect U247-51 had only weak and uncertain effects on the muscular contractions brought about by electric stimulation of the phrenic nerve, even though it was given in doses four to six times larger than those of tubocurarine, which produced a pronounced reduction

In the isolated rabbit heart (Langendorff) small doses of U247-51 (25-50 µg) slightly accelerated the perfusion without influencing frequency or amplitude Larger doses (200-250 µg) lowered both frequency and amplitude and had a rather varying influence on the perfusion rate Thus, no sympathomimetic

died The excitation was more pronounced in response to U247–51 than to U247–73, but the two quaternary compounds both caused greater excitation than the tertiary

As seen in the table, the four substances were approximately equally toxic when given intravenously After intraperitoneal and oral administration, on the other hand, the quaternary compounds were seen to be much more toxic than the tertiary In other words, the tertiary compounds were not in this way found to be absorbed to a greater extent than the quaternary from the gastro intestinal canal

Subcutaneous injections caused local irritation and in some cases produced necroses at the site of injection on prolonged treatment In tests on rabbits given trypan blue intravenously (HOPPE *et al* 1950) the quaternary compound U247–51 and the tertiary U247–125 had approximately equal locally irritating effects

2 *Chronic Toxicity of U247–51 and U247–73*

Rats were given 10–25 or 50 mg/kg orally once daily for two months

Mice were given 5 and 10 mg/kg i p and 25 and 50 mg/kg orally once daily through 3 months

All the treated animals thrived normally, and histological examination of the animals' organs revealed no pathological conditions

3 *Tests on Humans*

Three normal subjects received daily doses of 25, 50, and 75 mg U247–51 respectively through a period of 10 weeks

Haemoglobin, leucocyte count, thrombocyte count, and G P transaminase activity were recorded initially and again after 3, 7, and 10 weeks of medication

No significant alterations were observed during the test period, and the subjects reported no discomfort

A double-blind trial on ambulatory ulcer patients showed such favourable effects of both test substance and placebo that no significant difference could be noticed

4 *Tests on Isolated Organs*

As previously stated (ANTONSEN 1965a, 1967a), neither the quaternary nor the tertiary compounds were found to have any effects worth mentioning on the isolated *guinea pig intestine* Neither were their effects on the isolated *rabbit intestine* of any interest

In the isolated *rectus muscle* of the frog, on the other hand, antagonism to acetylcholine was demonstrated

is evidenced from the following investigations carried out at Mead Johnson's Pharmacological Laboratory, Evansville, USA

In anaesthetized cats which had had one side denervated, while the sympathetic nervous system could be stimulated in the other, injection of U247-51 effected contraction of the nictitating membrane in both sides In other words, no ganglion blocking action was seen Injection of bretylium, in doses large enough to obstruct liberation of noradrenaline at the nerve ending, prevented contraction of the nictitating membrane in response to nerve stimulation, whereas the reactions to adrenaline and U247-51 were increased Pretreatment of the cats with reserpine had no influence on the effect of adrenaline or U247-51, whereas no reaction was seen to nerve stimulation

Alpha receptor blockade by phenotolamine reduced the response to nerve stimulation and also prevented the activity of U247-51 and adrenaline (P M Lish 1965)

Gastric motility recordings by an open tube method revealed a rise in pressure concurrently with the pressor action No spasmolytic action was noticed, nor antagonism to the effects produced by carbacholine or barium chloride

In rats anaesthetized with urethane or a barbiturate U247-51 was found to produce a similar pressor action as in cats The effect was equally great in animals pretreated with large doses of reserpine

U247-73 may elicit a weak pressor action when given intravenously to rats or cats, much weaker than that obtained with the same dose of U247-51

The tertiary compounds have no characteristic effects on the blood pressure

7 *Effect on the Body Temperature*

The test substances could lower the normal temperature of mice and rats

The doses producing this effect depended on the experimental conditions Much larger doses were required on animals kept several together in closed boxes placed in a warm room than to animals kept single in open wire cages

The temperatures were measured with special applicators in connection with an electric universal thermometer (Electrolaboratory, Copenhagen)

The two quaternary compounds reduced the temperatures of rats when given intraperitoneally in doses of 5-10 mg/kg When given by stomach tube, about 10 times larger doses were required

The temperature-lowering effect of the tertiary compounds was obtained at 60-100 mg kg i p and at about 300 mg/kg orally

In mice intraperitoneal administration of U247-51, 2 5 mg kg and of U247-125, 100 mg kg, had definite hypothermal effects It was impossible to obtain reproducible hypothermia in mice with orally administered non-toxic doses of U247-51 and U247-125

effect was demonstrable These tests were, however, probably confounded by the irritative and surface-active effect of the substances

The isolated *guinea pig atrium* remained uninfluenced

In the isolated rat uterus no antagonism was seen to serotonin

In the isolated *vas deferens* of rats and guinea pigs (LEACH 1956) U247-51 intensified the actions of acetylcholine as well as of adrenaline and noradrenaline

5 *Tests for Analgesic Action*

The analgesic actions of the compounds were tested on mice by means of the hot-plate method, the phenylquinone method, and electric stimulation of the tail

After intraperitoneal injection both the tertiary and the quaternary compounds effected a marked rise of the pain threshold All the substances causing considerable local irritation, the prolonged reaction time may be due entirely or in part to diversion of the animals' attention owing to pain at the site of injection

The quaternary compounds were seen to have no effect when given orally in non-toxic doses to fasting mice The tertiary compounds ED50 were found to be about 500 mg/kg for U247-90 and about 300 mg/kg for U247-125 In other words, the effect was too weak as to be of any importance

6 *Effects on Anaesthetized Cats and Rats*

Effects of intravenous doses on blood pressure were tested on cats anaesthetized with a barbiturate or chloralose-urethane, and on cats subjected to spinal anaesthesia

U247-51 displayed an instantaneous, pronounced pressor action when given in doses of 0 5-2 mg The effect resembled that obtained with adrenaline, but was somewhat more prolonged It was not reduced by repeated injection of the same dose, nor by cutting off the sympathetic nerve or the vagus, nor by pretreatment with atropine, hexamethonium, chlorpromazine, or antazoline It could be reduced by pretreatment with dibenzyline

The pressor action of U247-51 was not altered by pretreatment with adrenaline, nor-adrenaline or tyramine Further, the compound had no influence on the depressor action obtained with acetylcholine or electric stimulation of the vagus nerve

Stimulation of the sympathetic nerve and recording of the contraction of the nictitating membrane revealed no ganglion-blocking effect of U247-51

That the pressure action provoked by injection of U247-51 is a direct sympathomimetic action—not produced by liberation of noradrenaline—

is evidenced from the following investigations carried out at Mead Johnson's Pharmacological Laboratory, Evansville, USA

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The temperature lowering effect of the tertiary compounds was obtained at 60–100 mg/kg i p and at about 300 mg/kg orally

In mice intraperitoneal administration of U247-51, 2 5 mg/kg, and of U247-125, 100 mg/kg, had definite hypothermal effects It was impossible to obtain reproducible hypothermia in mice with orally administered non-toxic doses of U247-51 and U247-125

Simultaneous measurement of the paw temperature revealed no significant alterations, neither in mice nor in rats

A considerable antipyretic effect was also obtained in rats with hyperthermia The hyperthermia had been elicited by subcutaneous injection 16 hours previously of *1 ml of a 20% suspension of dried brewers' yeast* (Levurine®) in water

Intraperitoneal injections of such doses which had effect on normal animals, (5–10 mg/kg) caused the body temperature of the febrile animals to fall from about 39° to about 35°, in other words to considerably below the normal temperature

Phenylbutazone, (100 mg/kg) included in comparative studies, lowered the temperature of rats with pyrexia to approximately normal, about 37 5°, *but had no effect in reasonable doses on the body temperature of normal* animals

Thus, the tests showed that the substances can bring about a hypothermia which cannot be ignored in the over-all assessment of their influence on the organism

8 *Tests for Sedative Effects on the Central Nervous System*

Tests for spontaneous activity on mice showed that *oral administration* of non toxic doses had no influence on the spontaneous activity of the animals

Pretreatment with the test substances was found to prolong the hexobarbitone induced sleeping time of mice However, the sleeping state could not be restored by giving the test substances to mice about to wake up This suggests that it is not a question of an increased sensitivity of the animals to hexobarbitone

Pretreatment with U247–51 and U247–73 could prolong the sleeping time of rats in response to phenobarbitone

10 mg/kg intraperitoneally caused a significant prolongation of the sleeping time after intraperitoneal injection of phenobarbitone

The effect was greatest when the interval between the two injections was 15 minutes, but still significant at intervals of 45, 90 and 120 minutes

The effect was approximately the same on male and female rats, and fairly independent of the dose Thus, there was no essential difference between the effects of 6, 12, 25, and 40 mg/kg

Pretreatment with U247–51 prolonged the chloral hydrate- and the alcohol-induced sleeping times of both mice and rats

These effects do not indicate that the substances possess any form of sedative action A barbiturate-intensifying action is a rather *unspecific* pharmacological one and the prolonged sleeping time is possibly due solely to the above-mentioned temperature-lowering action (FASTIER *et al* 1957)

9 *Corticotropin-Like Effect*

A possible corticotropin-like effect of these substances on rats was tested by the method indicated in Pharmacopea Nordica 1963 Determination of the ascorbic acid content in the adrenal glands of prednisolone-treated rats Corticotropin (Acton ®) was used for comparison

5 mg of U247-51 and U247-73 given intraperitoneally significantly reduced the ascorbic acid content of the adrenal glands (t≈3-4) The effect was greater than that of corticotropin (1 i u) injected subcutaneously

10 mg U247-51 given orally likewise had a marked effect (t≈1 7-2 0) The effect was smaller than that of corticotropin (2 i u) given subcutaneously

Finally, a test with 5 and 10 mg U247-51 and 80 mg U247-125 given orally showed that these doses lowered the ascorbic acid concentration to the same extent as 0 5 i u of corticotropin given subcutaneously

In a similar test on hypophysectomized animals 5 and 10 mg of U247-51 and U247-73 were administered orally The test substances were toxic on these animals So many of the treated animals died within the test period that relatively little importance can be attached to the results achieved with the remaining The ascorbic acid content of the adrenal glands seemed to become somewhat reduced in this group, too

The cause of the toxicity of the compounds on hypophysectomized animals is unknown

10 *Effect on the Blood Sugar*

As the inhibitory effects of the test substances on the gastric secretion might take place *via* an altered blood sugar level of the animals (Jow *et al* 1960, Roy 1964, Jordan & Quintana 1964, Aylett 1965), this effect was studied on rats

Pentobarbitone was used as anaesthetic, and the sugar concentrations were determined on blood obtained by cardiac puncture or blood drawn from the tail

Exploratory tests with intraperitoneal doses of U247-51 and U247-73 showed rises of the blood sugar level which might explain the effect on the stomach

However, more thorough investigations could not bear out this hypothesis The rats were found to have an exceedingly unstable blood sugar level, and a very extensive standardisation of the experimental conditions was therefore necessary The animals had to be anaesthetized prior to injection of the substances, because the very stress-effect of the locally irritating substances might elicit uncontrollable fluctuations of the blood sugar

Doses of the quaternary compounds which inhibit gastric secretion have no significant influence on the animals' blood sugar

Simultaneous measurement of the paw temperature revealed no significant alterations, neither in mice nor in rats

A considerable antipyretic effect was also obtained in rats with hyperthermia The hyperthermia had been elicited by subcutaneous injection 16 hours previously of 1 ml of a 20% suspension of dried brewers' yeast (Levurine®) in water

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induced irritative spots on the abdomen The interval to precipitation of dye in the spots gives a measure of the capillary permeability Both U247-51, 2 mg kg, and U247-90, 10 mg/kg delayed precipitation, while phenylbutazone, 20 and 50 mg/kg, did not

12 *Sympathomimetic Effects*

As stated in a previous section, U247-51 manifests a considerable pressor action when injected intravenously into anaesthetized animals

Further investigations were made to determine whether this indicates a sympathomimetic effect, which may have an influence in other tests

Action against reserpine induced ptosis

The estimations were based on RUBIN *et al*'s (1957) method

In mice U247-51, 25 mg/kg intraperitoneally, had a transitory effect This dose was less effective than 2 5 mg/kg of d amphetamine In other experiments U247-51, 20 mg/kg, was found to have less effect than 0 5 mg/kg of adrenaline No effect was observed of U247-73

U247-51 also had an antagonistic effect on reserpine induced ptosis *in rats* The two compounds had a common toxic effect (temperature-lowering?) which obscured the over all picture

As shown by GARATTINI *et al* (1960), an inhibitory action against reserpine-induced ptosis can be elicited by substances of very different types

Histamine aerosol treated guinea pigs

U247-51 had a marked antagonistic action against bronchospasms provoked in guinea pigs by histamine-aerosol The effect of 10 mg kg given intraperitoneally was highly significant and of the same order as that of 5 mg kg of antazoline (Antistina ®) This effect may be due to a sympathomimetic action since U247-51 has no antihistaminic effect on isolated guinea pig intestine U247-73 had no protective action against histamine-aerosol

Action against polymyxin induced ulcer in rats

The histamine liberator polymyxin B will provoke lesions in the stomachs of rats in the course of two hours Sympathomimetics, as well as antiserotonines and antihistamines counteract this activity (FRANCO-BROWDER 1959, MORENO & BRODIE 1962) ROBERT & NEZAMIS (1962) take the lesions to be of inflammatory origin and showed that corticoids have a protective action

U247-51 had a marked protective action when given in doses of 12 5-25 mg kg intraperitoneally or 25 mg kg orally U247-73 had no significant effect,

11 *Effect on Inflammation and Capillary Permeability*

The compounds under review have been tested for anti-inflammatory effect on rats by several methods Certain inflammatory processes were definitely inhibited The effect of orally administered U247–51 on œdema of the paws produced by carragenin was *twice as strong* as that of phenylbutazone, while the effect of U247–125 was about half as strong

In some tests a definite effect was obtained only by *injecting the substances* In such cases it is possible that the stress brought about by local irritation was responsible for part of the effect (CYGIELMAN & ROBSON 1963)

Various tests have been made using NEWBOULD's (1963) method of provoking chronic inflammation in rats by injecting killed Mycobact Tuberculosis U247–51 and U247–73 were seen to have no more than a limited effect on the primary inflammation, but a very definite inhibitory effect on the secondary, which affected the whole animal 10–12 days after the injection Only slight effects could be obtained with U247–90 Phenylbutazone had a considerably stronger effect against the primary inflammation than against the secondary U247–51, if given orally for two days prior to the injection, on the day of injection, and two days after (a total of five doses), also had a strong inhibitory effect on the secondary inflammation, setting in 8–10 days *after the last dose had been given Phenylbutazone administered over a similar* period had no inhibitory effect whatever on the secondary inflammation

The effect against cotton-pellet granuloma was tested by the method indicated by WINTER & PORTER (1957), according to which the animals are to be left untreated for 7 days after the implantation The test substances were mixed in the feed or given once daily with stomach tube It was not possible to find doses of U247–51 or U247–73 which reduced the granuloma weight without having a general toxic effect on the animals U247–125, 200 mg/kg, possibly had a weak effect

Erythema brought about by ultraviolet light on guinea pigs remained uninfluenced by the substances concerned The bronchoconstriction caused by bradykinin in anaesthetized guinea pigs also was not affected

Effects on capillary permeability were studied by two different tests

WILHELMI's (1950) method was employed for mice One ear was *irritated* by croton oil, while at the same time trypan blue was administered intraperitoneally Pretreatment *with substances reducing the capillary permeability* would postpone the time of distinct precipitation of dye in the ear *U247–73 and U247–51 were found to have strong effects*, and U247–90 also had a distinct effect All three compounds were more active than phenylbutazone, used for comparison

The method indicated by PARROT (1949) was employed in experiments on rabbits Animals given trypan blue intravenously develop chloroform-

induced irritative spots on the abdomen The interval to precipitation of dye in the spots gives a measure of the capillary permeability Both U247-51, 2 mg/kg, and U247-90, 10 mg/kg delayed precipitation, while phenylbutazone, 20 and 50 mg/kg, did not

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U247-51 had a marked protective action when given in doses of 12 5-25 mg kg intraperitoneally or 25 mg kg orally U247-73 had no significant effect,

while U247–125 was active only if given intraperitoneally in doses having a sedative effect on the animals

The results of the tests indicated the hypothesis that U247–51 possesses a sympathomimetic action, which can be demonstrated when the compound is administered otherwise than intravenously The previously mentioned effect on the capillary permeability is presumably due to the same mechanism of action

Such an action was not demonstrable of U247–73, nor of the tertiary compounds

13 *Effect on Gastric Emptying and Passage through Intestine*

Using the test meal method, we noticed (ANTONSEN 1965c) that U247–51 produced a delay of the gastric emptying, while U247–73 or the tertiary compounds did not

The two quaternary compounds had a laxative effect on mice and rats, both on parenteral and on oral administration

The influence of the test substances on the passage through the digestive system was further investigated by measuring the rate at which an orally administered carbon suspension passed through the gastro-intestinal canals of mice and rats

Several comparative investigations were made on mice, using the method indicated by JANSSEN & JAGENEAU (1957) The carbon suspension is administered into the stomach of the pretreated animals taking ten animals to each dose After a suitable interval the animals are killed by decapitation The number of animals in which the carbon has reached the cæcum is recorded

The results were as follows

U247–51, 0 625 and 1 25 mg/kg intraperitoneally, had a stimulating effect on the passage of carbon through the small intestine and provoked diarrhœa 2 5, 5, and 10 mg/kg reduced the rate of gastric emptying and delayed the passage through the small intestine Diarrhœa also occurred, however, in some instances after these doses, presumably owing to retention of water in the colon

U247–73, 1 25 mg/kg, promoted the passage of carbon through the small intestine and caused diarrhœa 2 5, 5, and 10 mg/kg effected no delay of the gastric emptying, but of the passage through the small intestine The compound had a similar laxative effect as U247–51

U247–125 delayed the passage through the small intestine in some animals, but had no influence on the gastric emptying nor any laxative effect

The effect on rats was tested as follows

30 minutes after pretreatment with test substance or sodium chloride

a carbon black suspension to which had been added a gum acacia mucilage was given by stomach tube to the fasting animals The rats were killed by a blow on the neck after different intervals, and the passage of the carbon through the intestine as well as the length of the whole intestine were measured

Table 2

The effects of U247-51 and U247-73 on the rates at which orally administered carbon passed through the digestive system of rats The figures represent mean values for 10 animals ± s e m

Pretreatment	Carbon passage in per cent of the length of the intestine		
	4 min	10 min	20 min
Control	52 4±8 6	65 9±5 9	73.2±5 0
U247-51 5 mg/kg	11 5±3 5	8 8±2 0	13 1+5.3
U247-73 5 mg/kg	16 1±3 3	36 8±5 0	32 7±8 6

The mean results are shown in *table 2* U247-51 had caused a marked delay of the passage of the carbon from the stomach into the intestine U247-73 likewise elicited a delay, but this was much less pronounced

The two quaternary compounds thus produced a delay in gastric emptying in mice and rats U247-51 had a much stronger effect than U247-73 Dependent on the dose, the compounds delayed or promoted the passage through the small intestine Diarrhœa was seen over the entire range of doses though not in every instance

The tertiary compound U247-125 only delayed the passage through the small intestine

14 *Influence on Absorption of Metropin®*

As stated in the introduction, U247-51 and U247-73 were prepared in the hope that they might stimulate the absorption of quaternary, anticholinergic compounds from the gastro-intestinal canal

This action was, in fact, noticed during the investigations on the rat stomach (ANTONSEN 1967a) It was further studied by measuring the influence of the substances on the mydriatic effect of Metropin® on mice The mydriatic effect has been employed as a criterion of absorption and duration of activity of anticholinergic substances (TONNESEN 1948, JAGENEAU & JANSSEN 1956, BRODIE 1966a)

This action has been employed for direct comparisons, even though recent investigations have shown that there is not necessarily parallelism between the effects of anticholinergic compounds on the stomach and their side-effects (KUBIN et al 1967a and b)

The procedure of the measurements have been described previously (ANTONSEN 1953, ANTONSEN & NIELSEN 1963) Oral doses were given by stomach tube to female mice, from which the food had been removed the night before

Neither U247-51 nor U247-73 elicited mydriasis in mice and the two substances had approximately the same effect in all the experiments

Table 3

Influence of U247-51 and U247-73 on the mydriatic effect of Metropin® on mice The test substances were given either simultaneously with or 10 minutes before or after Metropin®

Administration		Order of administration	Influence on the mydriatic effect of Metropin®
Metropin®	Test subst		
Orally	Orally	Together	Augmentation
Orally	Orally	Metropin® first	Augmentation
Orally	Orally	Test subst first	Delay of effect
Orally	i p	Test subst first	Delay of effect
i p	Orally	Test subst first	No influence
i p	i p	Metropin® first	No influence

Table 3 illustrates the influence of the test substances after oral and intraperitoneal administration They were given partly simultaneously with and partly 10 minutes before or after Metropin®

The pupillary diameters measured at suitable intervals during 6 hours indicated the onset, intensity, and duration of the mydriatic effect The result could thus be rendered graphically in the same manner as shown in *fig 1*

Only orally administered Metropin® was influenced by the test substances This indicates that only the absorption from the *gastro-intestinal tract* was influenced

The expected better absorption was seen when Metropin® was given prior to or simultaneously with the test substances When the latter were given first, their main effect was one of delaying the absorption This may be due to blocking of the routes of absorption of Metropin® The delay of the gastric emptying by the test substances may also have caused Metropin® to pass at a slower rate into the small intestine, from which it presumably is more readily absorbed than from the stomach

The influence on absorption is not due to alterations of pH, since Metropin® given orally in different buffer solutions with glycine and NaOH or HCl had the same effect as Metropin® dissolved in water

15 *Intravenous Infusion into Pretreated Mice*

As no peripheral effect was noticed of the test substances on the cholinergic nervous system, we tried to demonstrate a central effect.

Although the effect of acetylcholine on the gastric secretion generally is reckoned to be a purely peripheral one, ANICHKOV & GRECHISHKIN (1967) showed in experiments on dogs that a central mechanism of action has a certain influence on the gastric secretion.

The tests were made on mice, using the test method indicated by HINT & RICHTER (1958). After pretreatment with anticholinergics or parasympathomimetics, the test substances were infused slowly, while at the same time the reactions of the animals were observed until death occurred.

20 minutes before infusion the animals received intraperitoneally either 0.9% NaCl, atropine, Metropin®, or physostigmine. The test substances were then infused at concentrations which killed the controls within 5–10 minutes.

This was a somewhat slower infusion rate than that employed in the previously described tests for acute toxicity.

The mean results are shown in *table 4*. Physostigmine significantly reduced

Table 4

Lethal doses of the test substances administered by continued intravenous infusion to mice, pretreated 20 minutes before with NaCl, atropine sulphate, Metropin®, or physostigmine salicylate intraperitoneally. Mean values for six mice. t and p calculated on the basis of Student's test.

Pretreatment	Test substance	LD_{100}		t	p
		mg	mg/kg		
NaCl	U247-51	1.574	53.57		
Atropine 5 mg/kg	U247-51	1.208	44.10	1.0	0.1–0.5
Metropin® 20 mg/kg	U247-51	0.949	33.00	2.8	0.01–0.05
Physostigmine 0.5 mg/kg	U247-51	2.184	78.32	3.0	0.01–0.05
NaCl	U247-73	2.844	107.73		
Atropine 5 mg/kg	U247-73	3.140	107.30	0.02	>0.5
Metropin® 20 mg/kg	U247-73	2.716	91.53	0.81	0.1–0.5
Physostigmine 0.5 mg/kg	U247-73	4.289	151.36	2.3	0.01–0.05
NaCl	U247-125	4.672	142.2		
Atropine 5 mg/kg	U247-125	4.112	145.5	0.09	>0.5
Metropin® 20 mg/kg	U247-125	4.863	151.6	0.3	>0.5
Physostigmine 0.5 mg/kg	U247-125	8.043	267.3	3.9	<0.01
NaCl	U247-90	4.850	163.8		
Atropine 5 mg/kg	U247-90	5.929	180.1	1.0	>0.5
Metropin® 20 mg/kg	U247-90	5.335	173.7	0.03	0.5
Physostigmine 0.5 mg/kg	U247-90	9.130	269.1	2.3	0.01–0.05

The procedure of the measurements have been described previously (ANTONSEN 1953, ANTONSEN & NIELSEN 1963) Oral doses were given by stomach tube to female mice, from which the food had been removed the *night before*

Neither U247-51 nor U247-73 elicited mydriasis in mice and the two substances had approximately the same effect in all the experiments

Table 3

Influence of U247-51 and U247-73 on the mydriatic effect of Metropin® on mice The test substances were given either simultaneously with or 10 minutes before or after Metropin®

Administration		Order of administration	Influence on the mydriatic effect of Metropin®
Metropin®	Test subst		
Orally	Orally	Together	Augmentation
Orally	*Orally*	*Metropin® first*	*Augmentation*
Orally	Orally	Test subst first	Delay of effect
Orally	i p	Test subst first	Delay of effect
i p	Orally	*Test subst first*	*No influence*
i p	i p	Metropin® first	No influence

Table 3 illustrates the influence of the test substances after oral and intraperitoneal administration They were given partly simultaneously with and partly 10 minutes before or after Metropin®

The pupillary diameters measured at suitable intervals during 6 hours indicated the onset, intensity, and duration of the mydriatic effect The result could thus be rendered graphically in the same manner as shown in *fig 1*

Only orally administered Metropin® was influenced by the test substances This indicates that only the absorption from the gastro-intestinal tract was influenced

The expected better absorption was seen when Metropin® was given prior to or simultaneously with the test substances When the latter were given first, *their main effect was one of delaying the absorption* This may be due to blocking of the routes of absorption of Metropin® The delay of the gastric emptying by the test substances may also have caused Metropin® to pass at a slower rate into the small intestine, from which it presumably is more readily absorbed than from the stomach

The influence on absorption is not due to alterations of pH, since Metropin® given orally in different buffer solutions with glycine and NaOH or HCl had the same effect as Metropin® dissolved in water

some measure as a strong and persistent vagal stimulant The test substances may possibly have an antagonistic effect on such an action

PROCTOR *et al* (1967) have shown that amphetamine has an antagonistic effect on the central action of pilocarpine in mice There is, however, no evidence to suggest that a sympathomimetic action was of decisive importance in the results achieved from the present study, because if so, we should have expected a considerably stronger effect of U247-51 than of the other compounds

The infusion tests gave no further information which could help explain the mechanism of action of the test substances in the stomach No relationship was found between the actions noticed in these and those observed in the stomach tests We cannot, however, exclude the possibility that a quantitative difference between the substances may be concealed by a larger proportion of the tertiary compounds than of the quaternary entering the central nervous system

the toxicity of all the substances Metropin® augmented the toxicity of U247-51, but not that of the other substances, while atropine had no significant effect on any of the compounds

As stated in the section on acute toxicity tests, the intravenous infusion of the test substances caused considerable excitation at first No similar reaction was observed in the physostigmine-treated animals, in which only a sedative effect was noticed until the rising reflex was lost

That physostigmine acts as an antidote suggests a central anticholinergic action This is, however, inconsistent with the observation that atropine does not augment the toxicity

It has been shown, for instance, that atropine and other centrally acting anticholinergic substances have highly antagonistic effects on physostigmine injected intravenously into mice (CAZORT 1950) It is possibly a more peripheral action of the substances that is influenced by physostigmine That the toxicities of the tertiary and the quaternary compounds are influenced to equal extents, and that the quaternary Metropin® is more active than atropine both argue in favour of this view

After physostigmine had been found to have such a pronounced influence on the toxicity, the effect of pilocarpine was tested in the same manner As shown in *table 5*, this likewise reduced the toxicities of the two quaternary compounds and of U247-125, whereas it had less effect on U247-90

Table 5

Lethal doses of the test substances administered by continous intravenous infusion to mice pretreated 20 minutes before with NaCl or pilocarpine chloride intraperitoneally Mean values for six mice t and p calculated on the basis of Student s test

Pretreatment	Test substance	LD_{100}		t	p
		mg	mg/kg		
NaCl	U247-51	1 042	41 6		
Pilocarpine 5 mg/kg	U247-51	1 916	73 7	5 2	<0 001
NaCl	U247-73	2 313	88 1		
Pilocarpine 5 mg/kg	U247-73	3 398	132 4	4 2	<0 001
NaCl	U247-125	3 112	116 3		
Pilocarpine 5 mg/kg	U247-125	6 006	226 6	6 9	<0 001
NaCl	U247-90	4 815	172 4		
Pilocarpine 5 mg/kg	U247-90	5 291	198 9	1 5	0 1-0 5

Pilocarpine may prov[illegible] ...mal species including the rat (BASU MALLIK & [illegible]
and stimulates gastric secretion of pepsin and acid [illegible]

The test substances, which are salts of strong bases, will be fully ionized in the stomach, where accordingly they are unlikely to be absorbable (SCHANKER *et al* 1957, BRODIE 1964) To learn whether absorption takes place both proximal and distal to the ligation, a comparison was drawn between the effects of equal doses introduced into the ligated stomach and into the intestine just distal to the ligation respectively

Table 6

Effects of Metropin® and U247-95 on Shay rats Ligation period 5 hours The test substances administered orally in the ligated stomach or in the duodenum (i d) ȳ represents mean values for six animals t and p calculated on the basis of Student s test

		Metropin® 20 mg orally	Metropin® 20 mg i d	U247-95 2 mg orally	U247-95 2 mg i d
Volume ml	ȳ control	12 1	8 6	12 3	10 1
	ȳ test	11 6	1 7	7 2	3 9
	t	0 32	3 3	2 5	3 7
	p	>0 5	0 01–0 05	0 01–0 05	<0 01
pH	ȳ control	1 12	1 27	1 29	1 37
	ȳ test	1 28	3 31	1 51	1 99
	t	3 2	6 5	6 9	2 1
	p	0 01–0 05	<0 01	<0 01	0 01–0 05
Titrating pH 3 5 meq	ȳ control	1.220	0 816	0 962	0 723
	ȳ test	0 877	0 010	0 350	0 196
	t	2 6	3 4	6 4	2 9
	p	0 01–0 05	0 01–0 05	<0 01	0 01–0 05
Titrating pH 9 2 meq	ȳ control	1 363	0 938	1 131	0 866
	ȳ test	1 023	0 171	0 482	0 309
	t	2 3	3 0	6 2	2 9
	p	0 01–0 05	0 01–0 05	<0 01	0 01–0 05

Table 6 shows the results of a comparison between the effects of a quaternary and a tertiary anticholinergic compound in five hour ligated Shay rats

The quaternary Metropin® had a stronger inhibitory effect on the stomach when given into the intestine than when given into the ligated stomach, while very nearly the reverse was the case with regard to the tertiary compound U247-95

The control animals given 2 ml of water into the intestine presented a smaller volume and lower acid values than the controls given 2 ml of water into the stomach A calculation of the *t* values showed that the difference between the two control batches was on the border of being significant The 2 ml of water passed into the duodenum thus seemed to have an inhibitory effect In the previous experiments (ANTONSEN 1965a) it was doubtless a mistake not to include controls given water into the duodenum

Chapter 3

Influence of the Observed Pharmacological Properties on the Gastric Secretion of Rats

In previous papers (ANTONSEN 1965a,b,c, & 1967a,b,c) the author deals with the effects of the quaternary non-anticholinergic compounds on the gastric secretion of rats

After details have been given in the preceding chapter of the present work regarding other pharmacological properties noticed of the substances, the question naturally suggests itself which of these might be conceived to be responsible for the effect on the stomach

An attempt at answering this question has been made partly by reviewing the literature, and partly, where this was judged to be necessary, by supplementary examinations of the stomach

1 *Significance of the Quaternary Structure*

In a comparative study (ANTONSEN 1967a) was shown that the quaternary compounds have a stronger effect on the stomach than the tertiary

A direct comparison of these may be difficult, because the result will depend both on the mode of administration and on the duration of the test

Parenteral administration of the quaternary compounds gives a very imperfect picture of the effect to be expected after oral administration This is due partly to the very incomplete absorption from the gastro-intestinal tract, and partly to the fact that the substances may cause so much local irritation that the animals become exposed to a stress which may influence the result

Using the test meal method, oral administration at different points of time yields a measure of both intensity and duration of the effect (ANTONSEN 1965c)

In Shay-tests, however, the alimentary tract is blocked at the pyloric sphincter throughout the test period, which must necessarily extend over some hours

We tried to solve this problem (ANTONSEN 1965a, 1967a) by administering the test substances orally some hours before the ligation, or by passing them into the ligated stomach, or by injecting them direct into the duodenum or the cœcum

The results are shown in *fig 1* Metropin®, 50 mg/kg, had a considerably greater effect when injected into the intestine than when given orally U247-95, 10 mg/kg, also had a greater effect when administered into the intestine, but the difference from the effect of the same dose given orally was much smaller for this compound

The mydriatic test thus gave a fairly relevant picture of the effects of the two substances on the stomach in Shay-tests

Table 7

Effects of U247-51 and U247-125 on Shay rats Ligation period 5 hours The test substances given orally into the ligated stomach or injected into the duodenum (i d) Mean values for six animals Using Student s test, the effects of oral and intraduodenal administration are compared in test I In tests II and III the effects of the test substances are compared with those of H_2O given in the same manner

a indicates $p<0.1$ b indicates $p<0.05$ c indicates $p<0.01$

Test	Dose	Volume in ml	pH	Titrating values in meq	
				pH 3.5	pH 9.2
I	U247-51 2 mg orally	11.3	1.43	0.646	0.796
	U247-51 2 mg i d	7.2[b]	1.36	0.515	0.633
	U247-125 20 mg orally	10.5	1.41	0.617	0.817
	U247-125 20 mg i d	3.1[c]	2.62[b]	0.126[c]	0.211[c]
II	H_2O 2 ml orally	10.6	1.45	0.734	0.869
	U247-51 2.5 mg orally	9.7	1.68	0.371[a]	0.493[c]
	H_2O 2 ml i d	9.3	1.46	0.585	0.752
	U247-51 2.5 mg i d	3.9[c]	1.73[a]	0.183[c]	0.280[c]
III	H_2O 2 ml orally	11.3	1.37	0.744	0.882
	U247-125 20 mg orally	10.9	1.44	0.659	0.850
	H_2O 2 ml i d	7.8	1.42	0.555	0.672
	U247-125 20 mg i d	4.6[a]	2.23[c]	0.160[b]	0.299[a]

Table 7 shows the results of comparisons between the effects of U247-51 and the corresponding tertiary compound, U247-125, administered proximal and distal to the ligation in Shay rats

Test I was performed without use of control animals The quaternary compound here had a somewhat stronger effect when introduced into the intestine than into the stomach, but the difference was not significant

The tertiary compound, on the other hand, was significantly more active in the intestine than in the stomach ($p<0.01$)

In test II the effects of 2.5 mg U247-51 introduced into the stomach and into the intestine were compared with those of 2 ml H_2O given in the same manner to control animals After oral administration significant effects

A possible local action of the test substance introduced into the stomach may be another source of error According to BRODIE (1966b), anticholinergic substances possess such an action Finally, a part of the administered test substance may still remain in the stomach by the time the animal is killed and consequently influence the pH measurement and the results of titration

Tests have shown, however, that the pH values of the test solutions range from 4 5 to 6 5, and that titration of the whole amount administered requires no more than 5 to 10 per cent of the quantity of NaOH normally used during titration of stomach contents

The influence of the quaternary compounds on the absorption of Metropin® was assessed by means of the mydriatic effect of the substances on mice Metropin® and U247-95 were administered orally and into the duodenum Duodenal injections were given after laparotomy under light ether anaesthesia

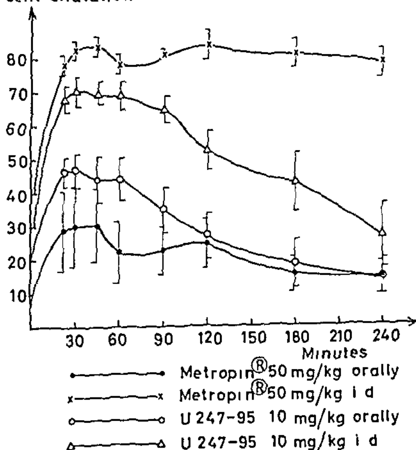


Fig 1 Mydriatic effects of Metropin® and 247-95 in mice The test substances given orally or injected into the proximal portion of the small intestine (i d) Abscissa Interval after administration *Ordinate* Dilatation of pupil stated in per cent of maximum pupil diameter Each curve represents the mean result for six mice, s e.m indicated by vertical lines

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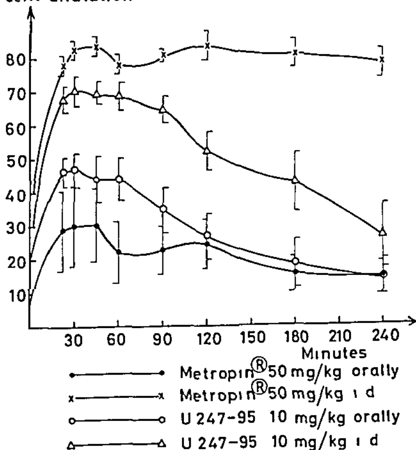


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In test III, a similar test with U247–125, the tertiary compound was considerably more active when introduced into the intestine than into the stomach

The experiments showed that use of Shay rats for testing the effects of these substances on the stomach after oral administration involves great difficulties

If the test substance is given orally so long before the test that it is certain to have disappeared from the stomach before the ligation, there is a risk that its effect has subsided within part of the test period

Absorption takes place both from the stomach and from the part of the alimentary tract situated distal to the ligation

We therefore cannot expect the test substance, if introduced proximal or distal to the ligation, to yield a correct picture of the absorption taking place in a normal alimentary tract after oral administration

Furthermore, the test showed that, dependent on the structure of the substance, considerable differences may be seen between the percentage absorbed from the stomach and that absorbed from the intestine

2 *Influence of the Pressor Effect*

As mentioned previously (ANTONSEN 1965c), the statements given in the literature concerning the effects of sympathomimetic substances on the gastric secretion are rather contradictory

FORREST & CODE (1954) take the inhibitory effect to be of vascular nature HARRIES (1957) on the other hand, is of the opinion that the effect is referable to an influence on the parasympathetic ganglia in the stomach wall DELANEY & GRIM (1965) and BASS & PATTERSON (1967) found sympathomimetic drugs to have an effect which was not proportional to the vaso active effect of the substances PRADHAN & WINGATE (1966) tried to assess the degree of the effects of sympathomimetic drugs on the stomach by blocking this effect with adrenergic blocking agents The latter proved, however, to have an independent effect interfering with the results JACOBSON in his review (1965) pointed out that very little is known about this relation, because we do not dispose of sufficiently reliable methods for measurements on non anaesthetized animals

Of the compounds tested in the present investigation only U247–51 had a sympathomimetic action strong enough to be considered of any importance

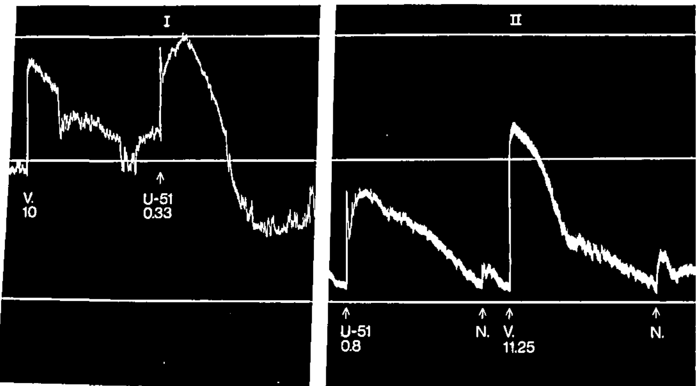


Fig 2 Blood pressure curves for two rats, both anaesthetized with chlorbutol II also treated with dibenzyline The three horizontal lines pass at 40-85 and 125 mm Hg All the doses were injected into the femoral vein V is the dose of vasopressin in m u U-51 is the dose of U247-51 in milligrams and N is 0 9 % NaCl

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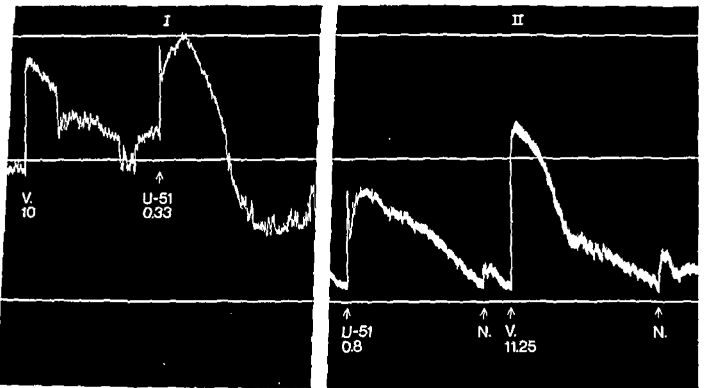


Fig 2 Blood pressure curves for two rats, both anaesthetized with chlorbutol *II also treated with dibenzyline* The three horizontal lines *pass at 40–85 and 125 mm Hg* All the doses were injected into the femoral vein V is the dose of vasopressin in m u U-51 is the dose of U247-51 in milligrams and N is 0 9 % NaCl

In test meal experiments the effect manifests itself by *a brief stimulation of the non-acid* gastric secretion and by a delay in stomach emptying (ANTONSEN 1965c)

In an attempt to clarify whether the pressor effect of U247-51 injected intravenously might be conceived to play a part, the effect of intravenous doses was studied in Shay-tests LD50 at intravenous injection into intact rats was *determined to 16 5 mg/kg for U247-51 and 14 5 mg/kg for U247-73*, and no essential differences were noticed between the visible reactions elicited by the two compounds in the animals

Vasopressin (Insipidin®) was used for comparison, because it produced blood pressure curves which best resembled those of U247-51 On direct comparison in chlorbutol anaesthetized rats the effect of 1 mg U247-51 was equal to that of 35 m u of vasopressin In rats treated first with dibenzyline 1 mg/kg i v (Blocadren®) 1 mg U247-51 had the same effect as 5 m u of vasopressin Dibenzyline reduced the effect of U247-51 without influencing *the vasopressin curves (fig 2)*

Table 8

Effects of U247-51, U247-73, and vasopressin on Shay rats Ligation period 5 hours The dose was given *i v immediately after the ligation Mean values for six animals* Using Student's test, the values for the treated animals were compared with those for controls in the same test

a indicates p < 0 1 b *indicates p < 0 05* c *indicates p < 0 01*

Substance		Volume ml	pH	Titrating values in meq	
				pH 3 5	pH 9 2
Control		7 8	1 27	0 682	0 823
U247-51	0 5 mg	5 6	1 79b	0 273b	0 428a
U247-51	1 0 mg	5 5	1 77	0 291a	0 435
U247-73	0 5 mg	5 8	1 89c	0 266a	0 446
U247-73	*1 0 mg*	*2 0*	*3 06c*	*0 032c*	*0 115c*
Control		9 0	1 41	0 729	0 896
U247-51	1 0 mg	4 4c	1 95a	0 193c	0 302c
U247-73	1 0 mg	2 4c	2 53c	0 051c	0 146c
Vasopressin	1 m u	10 9	1 48	0 837	1 002

8 lists the effects of 0 5 and 1 mg U247-51 and U247-73 and a of vasopressin (1 m u) on five-hour-treated Shay rats The dose ultaneously with the ligation Intravenously U247-73 showed ect than U247-51, while vasopressin had no demonstrable effect the effects of 1 mg U247-51 on two- and four-hour-ligated compared with the dose of vasopressin eliciting the same blood

Table 9

Effects of U247-51 and vasopressin on Shay rats Ligation periods 2 and 4 hours The dose given i v immediately after the ligation Mean values for six animals The results were compared by means of Student's test as stated under *table 8*

a indicates p<0 1 b indicates p<0 05 c indicates p<0 01

Substance	Period in hrs	Volume ml	pH	Titrating values in meq	
				pH 3.5	pH 9 2
Control	2	4 0	1 56	0 217	0 324
U247-51 1 mg	2	2 9	2 78[c]	0 055[a]	0 115[b]
Vasopressin 35 m.u	2	5 3	1 57	0 260	0 377
Control	4	9 1	1 17	0 851	1 010
U247-51 1 mg	4	5 9[a]	1 41[a]	0 402[c]	0 517[b]
Vasopressin 35 m u	4	8 8	1 28[a]	0 674	0 789

pressure rise in anaesthetized rats 35 m u While a significant effect of U247-51 was noticed both in the two- and in the four-hour experiment, vasopressin had no such effect In the two-hour experiment the vasopressin-treated animals displayed higher acid values and larger volumes than the controls, whereas in the four hour experiments a minor decrease was seen

These experiments showed that the pressor action elicited by intravenous injection had no influence on the inhibitory action of U247-51 on the stomach

Thus, the present experiments did not disclose such an inhibitory effect of the antidiuretic hormone on the gastric secretion which had been demonstrated by KARLMARK & ÖBRINK (1967) in Heidenhain dogs

As stated in chapter 2, a sympathomimetic action of U247-51 can also be demonstrated after oral or intraperitoneal administration However, the fact that U247-73 has a stronger effect on the stomach than U247-51 suggests that this action interferes only slightly with the effect on the stomach

In test meal experiments U247-73 had a stronger inhibitory effect than U247-51 on the spontaneous secretion and on histamine-stimulated secretion On the other hand, U247-51 had the strongest inhibitory effect on gastrin-stimulated secretion (ANTONSEN 1967c) Sympathomimetic action might accordingly here be conceived to play a certain role

3 *Influence of the Corticotropin Like Action*

COOKE (1967) in a review, states that although several workers have studied the influence of adrenocortical hormones on gastric function of rats, disagreement prevails concerning their activity, which, however, seems to be relatively low

MADDEN & RAMSBURG (1951) found no protective effect of corticotropin

in Shay rats WELBOURN & CODE (1953), in experiments on rats, noticed that cortisone or corticotropin had no effect on secretion nor on ulceration ROBERT & NEZAMIS (1958) observed that cortisone can protect rats against ulcers of the rumen, whereas it promotes ulceration in the body part of the stomach

BONTA (1961) found corticoid treatment to promote ulceration in Shay rats, but to moderate this process in rats exposed to stress

As reported in chapter 2, both U247-51 and U247-73, in doses known to inhibit gastric secretion, have a corticotropin-like effect The effect cannot be due to stress, because it can be elicited by both oral and parenteral administration

In direct comparative studies on Shay rats the effects of 0 5 and 5 i u of corticotropin (Acton®) were compared with that of 2 5 mg U247-51 While U247-51 had the usual inhibitory effect, both doses of corticotropin raised the acid secretion

In another experiment the same dose of U247-51 was compared with 2 × 5 i u of subcutaneously injected corticotropin, which had no demonstrable effect, and 2 × 20 i u of subcutaneously injected cortisone, which elicited a slight reduction of the secretion

Accordingly, there is no reason to believe that the demonstrated corticotropin-like effect of the test substances is a factor contributing towards their effect on the stomach

4 *Influence of an Anti-Inflammatory Effect*

It is known that anti-inflammatory substances often injure the stomach The exact mechanism of this action is unknown (DOMENJOZ 1966), but the effect is approximately proportional to the anti-inflammatory effect (PRABHU *et al* 1967) The injury which phenylbutazone, for instance, causes in the stomachs of rats will heal up spontaneously within few days, even if the administration of the substance is continued (BONFILS *et al* 1958, HORAKOVA *et al* 1965)

Even though an influence on secretion is not normally supposed to be responsible for the injury of the stomach, it has been shown that anti-inflammatory substances can reduce the gastric secretion in rats (METYS *et al* 1965, BONFILS *et al* 1954)

To study the effect of a typical anti-inflammatory drug on the secretion and gastric emptying of rats, phenylbutazone was given using the test meal method

Table 10 shows the effect of 20 mg phenylbutazone given orally The effect was tested both on the normal and on the histamine-stimulated secretion of the animals

Table 10

Effect of phenylbutazone on the gastric secretion in test meal experiments. 20 mg given orally 3 hours previously. In the experiments with histamine stimulation 1.5 mg histamine chloride was given immediately after the test meal, while in the experiments with spontaneous secretion 1 ml of NaCl v as injected intraperitoneally. N is the number of rats. Phenol red per cent is the percentage of test meal recovered at the end of the experimental period. V p p is the amount passing through the pyloric sphincter during the experimental period (45 minutes). These two figures indicate the rate of gastric emptying.

d is the difference between treated and control
t and p calculated according to Student's test

		N	pH	HCl µeq	Vol secr ml	V p p ml	Phenolred per cent
Spont secret	Control	20	3.07	65.8	2.16	4.66	28
	Phenylbut	19	3.09	50.7	2.26	4.09	36
	d		0.02	15.1	0.10	0.57	8
	t		0.1	1.4	0.3	1.9	0.7
	p		>0.5	0.1–0.5	>0.5	0.05–0.1	0.1–0.5
Histamine-stimul secret	Control	28	2.20	130.7	2.61	3.74	42
	Phenylbut	29	2.24	109.3	2.91	3.14	52
	d		0.04	21.4	0.30	0.60	10
	t		0.8	2.5	1.4	3.2	3.8
	p		0.1–0.5	0.01–0.05	0.1–0.5	<0.01	<0.01

A fairly large number of animals was required to obtain univocal effects. Following stimulated secretion phenylbutazone elicited a marked inhibition of the acid secretion without reducing the volume, as well as a marked delay of gastric emptying. Similar, though much less pronounced, effects were noticed on the spontaneous secretion.

The delay of *gastric emptying was the most* characteristic effect of phenyl-butazone on the rat stomach. Using other test methods this delay will more or less conceal the reduction of the acid secretion seen at the same time, a fact which possibly accounts for the weak and differing effects often observed.

The effect of phenylbutazone in test meal experiments is similar to that of U247-51 (ANTONSEN 1965c). We cannot exclude the possibility that the anti-inflammatory action of the test substances has a share in the effect of these compounds on the stomach.

However, the anti-inflammatory effect is no univocal concept. The investigations into this effect showed both quantitative and qualitative differences between the effect of phenylbutazone and those of U247-51 and U247-73.

Another possibility to be considered is that a local anti-inflammatory effect contributes towards preventing ulceration in the stomach. DOLL *et al*

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(1965) are of the opinion that the curative effect of carbenoxolone on gastric ulcers is of such a nature

5 *Significance of the Temperature-Lowering Effect*

Alterations of body temperature can doubtless influence both gastric secretion and ulceration in rats

In experiments on anaesthetized rats (ANTONSEN 1959) the importance of keeping the animals' temperature at a constant level was shown BRODIE & KUNDRATS (1964) noticed that a temperature fall of 2° caused a reduction of gastric secretion, and that the inhibitory action of morphine was proportional to its effect on temperature

RITCHIE (1967) showed that in rats, as in humans, the secretion can be greatly reduced for several weeks by a short-term, intense cooling down of the stomach

The temperature-lowering effect of our test substances on rats may have contributed to the reduction of gastric secretion However, the substances also had an inhibitory effect on gastric secretion when given in doses smaller than those which had a measurable influence on body temperature, as discussed in chapter 2

The temperature-lowering action of the test substances may have influenced development of gastric ulcers due to stress, which will be described in a future section BORNMANN (1965) noticed that in rats immobilisation ulcers were more easily provoked when the animals were left at a temperature of 18° than at 28° ROBERT *et al* (1966) likewise found that a high room temperature reduced the number of immobilisation ulcers developed

BUCHEL & GALLAIRE (1966) observed the same relationship between temperature and incidence of ulcer They also noticed that phenobarbitone and chloral hydrate could protect against ulceration, even though these drugs added to the fall of the animals' body temperature occurring during the experimental period

In our experiments with immobilisation ulcers the temperature-lowering effect of the substances may have been a contributory cause of the greatly varying results In animals left in open wire cages even a small dose of the test substances may cause a fall of the body temperature, which, as stated, will increase the liability to ulceration

6 *Possibility of a Central Effect on the Stomach*

The test substances having only slight peripheral effects, the idea naturally suggests itself that the effect on the stomach takes place *via* a central action on the animals

This question is, of course, closely related to the whole problem of the physiology of the rat stomach KOMAROV *et al* (1944) state that the gastric secretion of the rat is characterized by being histamine refractory, and by existence of a spontaneous secretion, which continues both during anaesthesia and during prolonged starvation The cephalic as well as the intestinal phase thus seem to be of minor importance LIN & ALPHIN (1958) arrived at the same result in experiments with chronic gastric fistula in rats In addition, they found that the rat has a powerful vagal tonus, which causes vagotomy to almost completely stop the secretion BRODIE *et al* (1962), by immobilizing both fistulated rats and Shay rats, showed that this stress could not stimulate the secretion in Shay rats, presumably because it already was maximal In fistulated rats the immobilisation raised the concentration of hydrochloric acid Anticholinergic drugs could prevent ulceration in both Shay rats and immobilized rats

The central effects of the test substances on the stomach were investigated partly through studies of their effect on gastric ulcers induced by stress, and partly in experiments on anaesthetized animals, in which the central factors must be supposed to have been eliminated

Stress induced ulcers

Rats exposed to stress of different kinds will develop ulcers of the glandular portion of the stomach (SELYE 1953) ROSSI *et al* (1956) involved a standardized method for inducing such ulcers by immobilisation of the animals for some hours This method has since been very extensively employed for studying the actions of drugs, because a certain aethiologic relationship is supposed to exist between these ulcers and those seen in clinical practice (HANSON & BRODIE 1960, BRODIE 1962)

Ulceration can be prevented by centrally acting drugs such as morphine (SIMLER *et al* 1962) thalidomide (MARTINDALE *et al* 1960) and chlorpromazine (MENGUY 1960, BONFILS *et al* 1961) BONFILS *et al* (1963) performed a very comprehensive investigation into the effects of psycho pharmacological drugs The animals were protected by anticholinergic drugs as well as by vagotomy (BONFILS *et al* 1961, BRODIE *et al* 1962)

We have previously employed the method in experiments where we found a quaternary atropine derivative to be more active than atropine (ANTONSEN & NIELSEN 1963)

Tests with U247-51 and U247-73 administered intraperitoneally or subcutaneously gave greatly varying results In some tests a protective effect was seen, but equally often a more pronounced ulceration was found than in controls

Given by stomach tube 10 mg of U247-51 and U247-73, and 100 mg

of U247-125, were found to have a weak protective effect. The effect was much less stable than that of the quaternary, anticholinergic compound U247-96, which was included in comparative studies.

The test substances doubtless have a certain protective effect against gastric ulcers induced by stress. However, when given parenterally their locally irritating and sympathomimetic actions aggravate the stress to which the animals are exposed. As stated previously, the temperature-lowering effect of the substances can likewise intensify the tendency to ulceration. After oral administration the amount absorbed may be too small to demonstrate a definite effect.

Experiments with anaesthetized rats

The method employed in these experiments was developed to improve the measurement of gastric secretion in anaesthetized rats (ANTONSEN 1959).

The principle of the method is that of inserting a dialysis bag filled with water in the stomach of the anaesthetized rat. The hydrochloric acid secreted in the stomach will dialyse into the bag. By changing the contents of the bag every 5 minutes we get an excellent measure of the amount secreted. The concentration of acid in the aspirated fluid is measured both by determining the pH with glass electrode, and by titration with 0.01 N NaOH. Electrometric titration to pH 9.2 has been employed instead of the previously used titration with phenolphthalein as indicator.

In the first study the method was shown to be suitable for measuring variations in the spontaneous acid secretion of the rat and for measuring the stimulating effect of histamine. The method proved, however, to be less suitable for measuring the effect of antagonists.

The spontaneous acid secretion was found to be reduced after 250 and 500 μg of both Metropin® and the quaternary compounds. It was difficult to obtain a quantitative measure of the effect. This was because it sometimes took a long time before a given dose ceased to have effect so that another could be given, and even if the effect apparently had ceased, there might be an after-effect on the following dose. The inhibitory effect of the quaternary compounds on gastric emptying is another source of error. Using the above technique this factor will cause a minor proportion of the secreted acid to pass out through the pyloric sphincter and presumably dialyse instead through the dialysis bag. Finally, the pressor action which can be elicited by intravenous injection of U247-51 may cause a brief enhancement of secretion (GHOSH & SCHILD 1958, ANTONSEN 1959).

Atropine, which, as shown previously (ANTONSEN 1965b), does not have the same delaying effect on gastric emptying as the quaternary compounds,

inhibited secretion to such an extent that we never succeeded in getting it started again without stimulation

Both Metropin® and the quaternary compounds demonstrated an antagonistic effect on the stimulation of acid secretion by single doses of histamine A quantitative comparison of different doses on the same rat is difficult, because it takes a long time before a given dose has lost its effect It has been shown previously, by means of the test meal method (ANTONSEN 1965b), that the mydriatic effect of Metropin® reaches maximum after a short while, whereas the maximum effect on the stomach is not attained till after 5 hours U247-51 and U247-73 have maximum effect after about 3 hours

In a few experiments we tested the effect of the test substances on secretion stimulated by continous intravenous infusion of histamine The animals were given from 0 4 to 2 0 mg histamine dissolved in 2 ml of 0 9% NaCl per hour No more than a weak antagonistic effect was noticed of the test substances, and it was difficult to find a histamine dose giving a constant acid secretion for some length of time that did not have a toxic effect on the animals

Metropin® had an antagonistic effect on metacholine stimulated secretion, which offered the same difficulties as the histamine stimulated, whereas U247-51 had no such effect

The results of these investigations suggest that the test substances have no central effects interfering definitely with their inhibitory effect on the gastric secretion

A marked inhibitory effect was demonstrable in anaesthetized animals, whereas the effect against stress induced ulcers was slight

The central effects of the substances seemed rather to act contrary to expectation in these experiments Thus, the body temperature of the animals was lowered, a fact which would make stress-rats more liable to ulceration

A sympathomimetic effect was seen, which would add to the excitation and consequently the stress during the immobilisation Finally, gastric emptying was delayed, causing the acid produced during the immobilisation to remain longer in the stomach

Also, the barbiturate intensifying action mentioned earlier cannot be regarded as a sign that the test substances have any form of central sedative action

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Chapter 4

Conclusion

The present investigations gave results showing that the test substances possess none of the properties normally explaining an inhibitory effect on the stomach

The following are the most important actions observed

1. An anticholinergic action *in vitro* of U247–51, whereas not of U247–73, on the rectus muscle of the frog
2. A sympathomimetic action of U247–51, demonstrable in blood pressure tests on anaesthetized animals, and in various other tests
3. The test substances induce hypothermia in normal mice and rats, as well as in rats with hyperthermia
4. They can intensify the effects of barbiturate, alcohol and chloral hydrate on mice and rats
5. They have a corticotropin-like effect on prednisolone-treated rats
6. An anti-inflammatory and a capillary-permeability-reducing effect have been demonstrated in certain tests
7. They have such an intense locally irritating action that a stress factor may be involved when they are administered parenterally
8. They delay gastric emptying, influence the rate of passage through the intestine, and may cause diarrhœa
9. They can stimulate the absorption of a quaternary, anticholinergic compound, when this is given before or simultaneously with the test substances into the stomach
10. The toxicity at intravenous infusion into mice is reduced by pretreatment with physostigmine or pilocarpine Pretreatment with Metropin® adds to the toxicity of U247–51 but not to that of U247–73

None of these actions can alone explain the effects of U247–51 and U247–73 on the stomach, and it is worth noting that U247–51 had the strongest effect in most tests, but that U247–73 had the strongest effect on the stomach

Some of the actions observed must be supposed to have influenced the measured effects on the stomach A sympathomimetic, a temperature-lowering,

an anti-inflammatory, and an increased capillary-permeability. The gastric effects were observed with dose levels which did not elicit the above mentioned actions.

Stress due to local irritation on injection was a source of error in the first tests for effect on the blood sugar level and may also have played a role in other tests. HAROUTUNIAN *et al.* (1952) found the Shay test to be rather unspecific, and noticed a protective effect of turpentine oil given intraperitoneally. SEGAL *et al.* (1952) found various substances to have a protective effect which was proportional to their toxicity. We have therefore always tried to verify the results achieved after parenteral administration with those of experiments with oral administration. The relatively low absorption of the quaternary compounds from the gastro-intestinal tract will thus come into the picture. The substances may very well have effects which do not manifest themselves on parenteral administration owing to a concurrent stress effect, and are not detectable by oral administration either owing to absorption of a very small amount only.

Furthermore, there is, of course, a possibility that the compounds act in a manner of which we have not been aware.

A specific inhibitory effect on pepsin activity might exist, an effect which would also be able to prevent ulceration in Shay rats (COOK & BIANCHI 1967). However, pepsin activity measurements carried out on rats treated with the test substances have always disclosed a close proportionality between reduction of volume and pepsin activity.

It has previously been mentioned that the commonly employed gastric-secretion inhibiting drugs are active in tests on rats. However, substances of totally different types have also been reported to have effect. SEMB (1966) found a preparation gained from the antrum of dogs to have a considerable inhibitory effect on Shay rats—a mechanism of action which presumably can be left out of account in the present study. BASS *et al.* (1966) examined some substances whose effect on rats bore a certain resemblance to that of the test substances described in the present paper. These substances likewise do not act *via* the cholinergic nervous system. The authors are of the opinion that a sympathomimetic action is the most likely mechanism. BLUM *et al.* (1967), in a study on the effect of cholinergic drugs on Shay rats, noticed that while peripherally acting, cholinergic drugs promoted ulceration, the centrally acting cholinergic drug tremorine had an inhibitory effect.

The other problem which we hoped to solve through the present investigations was whether the methods commonly employed in rat experiments are relevant.

This problem could, of course, best be solved by testing the effect of the substances on other animal species and on humans.

In cats anaesthetized with chloralose-urethane and provided with a stomach

fistula U247–51 injected intravenously or intramuscularly was demonstrated to inhibit histamine-stimulated secretion

In our laboratory no investigations have been made into the effects of the test substances on the gastric secretion of dogs In the Wyeth Laboratories ROSENTHALE (1966) observed an unexpectedly pronounced toxicity of both U247–51 and U247–73 when these were given orally to dogs Vomiting and salivation followed after 1 mg per kilogram, excessive vomiting after 10 mg/kg, while after 25 mg/kg the vomit was blood-stained Owing to this strong emetic effect on dogs secretion tests on these animals were abandoned

The above-mentioned dialysis method was originally developed for use on humans (MARNER 1958) The method has been employed in collaboration with Dr Marner for measuring the influence of the test substances on the gastric secretion of normal volunteers

The test substances were given orally at different point of time before histamine stimulation

In some of the experiments a certain inhibitory effect was demonstrated of U247–51 and of Metropin®, but no quantitative estimation was obtainable

It has, in other words, been impossible to attain to an evaluation of the rat experiments on the basis of experiments with other animal species or humans

The present investigations have plainly shown that the inhibitory effect noticed on the gastric secretion of Shay rats after injection of a test substance gives very little information on the general pharmacological action of the substance concerned Such a test is only of value for a rough screening of a large number of substances and for a quantitative comparison of substances having the same mechanism of action

The Shay-test is less fit for studying the effect of an orally administered test substance Nevertheless, certain data concerning this effect are obtainable, if a suitable experimental technique is employed

Using the Shay-test we always found agreement between the effect on the secretion in short term tests and the effect on ulceration in more prolonged tests This is consistent with the pathogenesis of the ulcers originally suggested by SHAY *et al* (1945), namely a corrosion of the parts of the stomach which are not normally exposed to the action of an acid LAMBLING *et al* (1953), however, advanced the hypothesis that the ulcers developed in Shay rats are a manifestation of a diffuse visceral disease arising as a result of irritation of the pyloric sphincter In addition to these two theories regarding the pathogenesis of ulceration in Shay rats, other factors have been pointed out as important, such as the electrolyte loss with the gastric juice (KOWALEWSKI *et al* 1954), and the vasomotor reactions for which the ligation is responsible (DEBRAY *et al* 1950, GATI *et al* 1961, LOZZIO *et al* 1961, JOHANSSON & NYLANDER 1964)

The test meal method gives many more details regarding the effect of a test substance on the gastric secretion

The inhibitory effect of the test substances noticed in these tests was in fair agreement with the results of the Shay test, but considerable differences were seen between the effect on the volume and that on the acid secretion

Furthermore, the method showed that U247-51 causes a considerable delay of the passage from the stomach to the intestine, whereas U247-73 has no such effect (ANTONSEN 1965c) A measurement of this delaying action is doubtless a very important factor in the evaluation of the effects of test substances on the stomach HAFTER (1965), for instance, is of the opinion that the effect of an anticholinergic substance on secretion is of less consequence than its effect on the tone and motility of the stomach SCHAPIRO *et al* (1965) showed that there need not be a correlation between the effect on the motility and that on the secretion On combined treatment with an antisecretory agent and an antacid drug a delay of the emptying will likewise be an important factor in the total effect In the comparative studies of the effects of gastrin and histamine (ANTONSEN 1967c) test meal experiments showed a difference between the effects of these two drugs on the gastric emptying, a fact which may influence the result of the stomach function test

The test meal method has also been employed on humans (HUNT & SPURRELL 1951, HUNT 1959), but on no other animal species than the rat It would doubtless be unsuitable for dogs, because such frequent passing of a tube through the œsophagus would cause a confounding emesis in these animals

The method has the great advantages that anaesthesia and surgical intervention are avoided, and that cross-over tests can be conducted

The test substances can be given orally at different points of time before the test This affords a possibility of measuring both the duration of their effect and the time of maximum effect By means of such tests remarkable differences have been found between the point of time at which anticholinergic substances manifest a mydriatic action and that at which they act on the stomach

We may conclude from the results of the present investigations that the rat when used in a suitable manner, can serve to give valuable information on the effect of a new drug on the stomach Even greater benefit will presumably be derived from the rat as experimental animal for such investigations by also using rats with a chronic stomach fistula (LIN & ALPHIN 1958, HALTER *et al* 1966, HÅKANSON *et al* 1967) Having too little experience with regard to this method, we are still unable to make any valuation of it

Our original intention with the test substances was to test whether they might promote the absorption of quaternary, anticholinergic compounds from the gastro-intestinal canal

Such an effect has been recorded, it is true, but we found the substances to have so many other effects that their use may be feared to involve a risk of considerable side-effects

The question then suggested itself whether an attempt should be made to prepare pharmacologically less active substances with the same stimulating effect on the absorption However, the studies made on mice regarding the influence of the test substance on the absorption of Metropin® gave results indicating that the degree of this effect will depend on various factors that are difficult to control

Summary

Introduction

The present work is a continuation of previous studies of some quaternary, non anticholinergic compounds having an inhibitory effect on the gastric secretion of rats

Its purpose was to clarify, if possible, the mechanisms of action of the compounds, and partly to obtain a greater knowledge of the applicability of the rat for studies of the stomach

Chapter 1

Chemical Configuration of the Tested Compounds

Two quaternary, non anticholinergic compounds U247–51 and U247–73, as well as the corresponding tertiary compounds U247–125 and U247–90 were studied In some of the tests the quaternary, anticholinergic compound U247–96 and its tertiary analogue U247–95 were also used Finally, as in previous studies, Metropin ®, a quaternary, anticholinergic compound used in clinical work, was included

Chapter 2

Pharmacological Studies of the Quaternary,
Non Anticholinergic Compounds

1 The acute toxicities of the quaternary and the tertiary compounds were studied in experiments on female mice We employed intravenous, intraperitoneal, and oral administration, as well as continuous, intravenous infusion
2 Rats were used for chronic toxicity studies on the quaternary compounds No injuring effect was demonstrable in these animals
3 U247–51 was given to three normal subjects for 10 weeks without causing side effects
4 In experiments on isolated organs the substances had no effect in the majority of the cases An anticholinergic action was demonstrated in the rectus muscle of frogs, while the vas deferens of rats and guinea pigs showed an intensification of the actions of acetylcholine, adrenaline, and nor adrenaline
5 Orally administered substances had no analgesic effect on mice

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2 Rats were used for chronic toxicity studies on the quaternary compounds No injuring effect was demonstrable in these animals

3 U247-51 was given to three normal subjects for 10 weeks without causing side-effects

4 In experiments on isolated organs the substances had no effect in the majority of the cases An anticholinergic action was demonstrated in the rectus muscle of frogs, while the vas deferens of rats and guinea pigs showed an intensification of the actions of acetylcholine, adrenaline, and nor adrenaline

5 Orally administered substances had no analgesic effect on mice

6 In anaesthetized cats and rats U247-51 elicited an adrenaline-like pressor action, which seemed to be a direct sympathomimetic action No similar action was seen of U247-73 nor of the tertiary compounds

7 The test substances lowered the rectal temperature in normal rats and mice as well as in rats with pyrexia The degree of this effect was found to depend on the form of administration and on the environment of the animals

8 In tests for a sedative central action the test substances had no influence on the spontaneous activity of mice On the other hand, these substances effected a prolongation of the sleeping times of mice and rats in response to barbiturate, chloralhydrate and alcohol

9 The test substances had a corticotropin-like effect on prednisolone-treated rats, both after parenteral and after oral administration In hypophysectomized animals their action was so toxic that the tests could not be carried through

10 The blood sugar was seen to remain uninfluenced by oral doses equal to those inhibiting gastric secretion Given intraperitoneally, the test substances increased the blood sugar level, presumably owing to an irritative action

11 The test substances had an appreciable inhibitory effect on inflammatory processes provoked by carragenin or killed bacterial cultures Cotton-pellet granuloma tests on rats and ultraviolet tests on guinea pigs, on the other hand, revealed no effect The test substances reduced the capillary permeability in both mice and rabbits

12 U247-51 had an antagonistic effect on reserpin-induced ptosis in mice, on bronchospasms produced by histamine-aerosol in guinea pigs, and on polymyxin-induced ulcer in rats These effects are presumably accountable for by a sympathomimetic mechanism of action Neither U247-73 nor the tertiary compounds had similar effect

13 After oral administration of a carbon black suspension the test substances delayed gastric emptying in mice and rats, caused diarrhœa, and, dependent on the dose, delayed or accelerated the passage through the small intestine

14 By measuring the mydriatic effect on mice the test substances were shown to be able to stimulate the absorption of the quaternary, anticholinergic substance Metropin® from the gastro-intestinal canal

The effect was seen only when Metropin® was given before or simultaneously with the test substances

15 Pretreatment with physostigmine or pilocarpine reduced the toxic effects on mice of intravenously infused test substances Pretreatment with atropine had no such effect, while Metropin® added to the toxicity of U247-51

CHAPTER 3

Influence of the Observed Pharmacological Properties on the Gastric Secretion of Rats

1 The quaternary compounds have been shown previously to have a stronger effect on the stomach than the tertiary

Using the test meal method, the test substances can be given orally at different times before the measurements However, oral administration to Shay rats is difficult Comparisons between quaternary and tertiary, anticholinergic and non-anticholinergic compounds showed that absorption takes place during the test period, both from the ligated stomach and from the rest of the alimentary tract The ratio of the amounts absorbed on either side of the ligation depends on the nature of the test substance

Injection of water into the duodenum elicits an inhibitory effect on the gastric secretion

2 To study the blood pressure raising effect after intravenous injection U247-51 was compared with a dose of vasopressin having the same influence on blood pressure U247-51 had a considerable inhibitory effect in two- and four-hour Shay-tests, the vasopressin doses used had no demonstrable effect

3 Direct comparisons made on Shay rats showed that neither corticotropin nor cortisone had any inhibitory effect on the gastric secretion

The observed corticotropin like effect of the test substances is therefore unlikely to have influenced the effect on the stomach

4 The effect of phenylbutazone on gastric secretion and emptying was studied in test meal experiments with and without histamine stimulation The substance was seen to inhibit secretion and also to a very great extent the passage from the stomach to the intestine

Although there are considerable differences between the effect of phenylbutazone and those of U247-51 and U247-73 in tests for anti-inflammatory action, we cannot exclude the possibility that an anti-flammatory or capillary action may be involved in the effect of the test substances on the stomach

5 The temperature-lowering effect of the test substances may in certain experiments have contributed to the measured effects on the stomach

6 As the test substances have no peripheral actions that might account for their inhibitory effects on the stomach, this inhibition may be due to a more central action on the animals

The test substances had, however, a very unstable effect on gastric ulcers induced by emotional stress In anaesthetized rats, on the other

hand, an inhibitory effect was demonstrable both on the spontaneous gastric secretion and on histamine-stimulated secretion

These experimental results suggest that the test substances possess no central actions having any definite share in their inhibitory effect on the gastric secretion

Chapter 4

Conclusion

The most important ones of the observed pharmacological actions of the test substances have been collected in ten items

None of these actions can alone be responsible for the effect on the stomach A sympathomimetic action, as well as a temperature-lowering and an anti-inflammatory action are likely to have had a certain influence on the results

The difficulty of clarifying the mechanism of action of the substances is doubtless in some measure due to the incomplete absorption from the gastro-intestinal canal and to irritation on parenteral administration

A review of the literature revealed no description of stomach-active substances that might have acted in the same manner as the tested compounds

The problem whether the methods generally employed for testing the reactions of rat stomachs can give relevant results can only be settled by comparative studies on other animal species and humans

Intravenously injected U247–51 had an inhibitory effect on the gastric secretion of anaesthetized cats Similar experiments on dogs were abandoned, because the test substances had an emetic, toxic effect on these animals Experiments on human volunteers revealed a certain inhibitory effect on the gastric secretion Quantitative comparisons have been impossible to carry through, however

The experiments as well as a review of the literature showed that an inhibitory effect elicited by injecting a new drug into Shay rats is a highly unspecific criterion of effect The Shay-test is suitable, on the other hand, for quantitative comparisons of known drugs Testing the effect of an orally administered substance presents considerable difficulties, because the alimentary tract is bloked below the stomach

The test meal method is much better suited for testing an orally administered substance Furthermore, this method can give valuable information on the influence of a test substance on the rate of gastric emptying

The test substances were prepared for the purpose of trying to stimulate the absorption of quaternary, anticholinergic compounds from the gastro-intestinal canal However, the results achieved so far suggest that they will have too many side-effects to be useful for this purpose

References

(World Medical Periodicals 1961)

Anichkov, S V & L L Grechishkin Influence of acetylcholine and benactyzine on central cholinergic structures regulating gastric secretion *Arch int Pharmacodyn* 1967, **166**, 417–423

Antonsen, S Comparison of the atropine-like actions of some quaternary ammonium compounds and their effects on "Shay rats" *Acta pharmacol et toxicol* 1953, **9**, 47–58

Antonsen, S The influence of sex hormones on experimentally produced gastric ulcer in rats *Acta endocr* (Kbh) 1955, **19**, 203–208

Antonsen, S Determination by dialysis of spontaneous and histamine-stimulated acid secretion in the rat stomach *Acta pharmacol et toxicol* 1959, **16**, 78–86

Antonsen, S Some quaternary, non anticholinergic compounds with an inhibitory action on 'Shay rats' *Acta pharmacol et toxicol* 1965a, **23**, 154–164

Antonsen, S Gastric secretion of rats after test meals *Acta pharmacol et toxicol* 1965b, **23**, 165–178

Antonsen, S Application of test meal method for investigating the effect of some quaternary non anticholinergic substances on the rat stomach *Acta pharmacol et toxicol* 1965c, **23**, 179–188

Antonsen, S Comparison of the inhibitory effects of tertiary and quaternary compounds on the gastric secretion of rats *Acta pharmacol et toxicol* 1967a, **25**, 405–419

Antonsen, S Comparison of the stimulating effects of gastrin and histamine on the gastric secretion of rats *Acta pharmacol et toxicol* 1967b, **25**, 420–426

Antonsen, S Antagonism of gastrin- and histamine-stimulated gastric secretion in rats. *Acta pharmacol et toxicol* 1967c, **25**, 427–434

Antonsen, S & G H Nielsen A quaternary atropine derivative *Acta pharmacol et toxicol* 1963, **20**, 357–366

Aylett, P Effects of tolbutamide upon gastric secretion and emptying *Brit med J* 1965, **1**, 1464–1466

Bass, P & M A Patterson Gastric secretory responses to drugs affecting adrenergic mechanisms in rats *J Pharmacol exp Ther* 1967, **158**, 142–149

Bass, P, R A Purdon & M. A Patterson Gastric antisecretory and other pharmacologic studies of 3-methylamino-2, 1-benzisothiazole *J Pharmacol exp Ther.* 1966, **153**, 292–300

Basu Mallik, K C & S K Gubta An experimental study of gastric ulcers produced by pilocarpine *Pathophynology of peptic ulcer* McGill University Press 1963 301–309

Blum, H, E Mutschler & P A. van Zwieten Über den Einfluss von cholinergen Pharmaka auf die Ulcusbildung und die Magensaftsekretion bei der Shay-ratte *Naunyn-Schmiedeberg's Arch exp Path Pharmak* 1967, **256**, 99–111

Bonfils, S, M Dubrasquet & A Lambling Ulcère expérimental de contrainte *Therapie* 1961, **16**, 384–392

Bonfils, S, M Dubrasquet & A. Lambling Dérivés de la phénothiazine et de l'imin-

odibenzyle relation dose-efficacité vis-à-vis de l'ulcère expérimental de contrainte *Thérapie* 1963, **18**, 373–389

Bonfils, S, J P Hardouin & Fl Delbarre Ulcération gastrique du rat blanc par administration de phénylbutazone *C R Soc Biol (Paris)* 1954, **148**, 881–884

Bonfils, S, J P Hardouin, C Richir & A Lambling Lésions gastriques du rat blanc par administration de phénylbutazone étude de quelques facteurs de "protection" *Thérapie* 1958, **13**, 490–501

Bonta, I L A study of the effect of some glucocorticoids and A C T H on artificially induced gastric ulcers of the rat *Arch int Pharmacodyn* 1961, **132**, 147–163

Bornmann, G Studie uber das sogenannte Immobilisationsulcus *Naunyn-Schmiedeberg's Arch exp Path Pharmak* 1965, **251**, 200

Brodie, B B Physico-chemical factors in drug absorption *Absorption and Distribution of Drugs* E & S Livingstone Ltd 1964 pag 16–48

Brodie, D A Ulceration of the stomach produced by restraint in rats *Gastroenterology* 1962, **43**, 107–109

Brodie, D A A comparison of anticholinergic drugs on gastric secretion, gastric emptying, and pupil diameter in the rat *Gastroenterology* 1966a, **50**, 45–50

Brodie, D A The mechanism of gastric hyperacidity produced by pylorus ligation in the rat *Amer J dig Dis* 1966b, **11**, 231–241

Brodie, D A & S K Kundrats The effect of pyrexia on rat gastric secretion *Gastroenterology* 1964, **47**, 171–178

Brodie, D A, R W Marshall & O M Moreno Effect of restraint on gastric acidity in the rat *Amer J Physiol* 1962, **202**, 812–814

Buchel, L & D Gallaire Influence de la température ambiante sur la production d'ulcères de contrainte chez le rat *C R Soc Biol (Paris)* 1966, **160**, 1817–1820

Cavallito, C J & T B O'Dell Modification of rates of gastrointestinal absorption of drugs II Quaternary ammonium salts *J Amer pharm Ass* 1958, **47**, 169–173

Cazort, R J Atropine-like action of alpha, alpha-diphenyl-gamma-dimethylaminovaleramide HCl (BL 139) *J Pharmacol exp Ther* 1950, **100**, 325–332

Code, C F Histamine and gastric secretion a later look, 1955–1965 *Fed Proc* 1965, **24**, 1311–1321

Cooke, A R Role of adrenocortical steroids in the regulation of gastric secretion *Gastroenterology* 1967, **52**, 272–281

Cook, D L & R G Bianchi SC-15396 A new antiulcer compound possessing antigastrin activity *Life Sciences*, 1967, **6**, 1381–1387

Cygielman, S & J M Robson The effect of irritant substances on the deposition of granulation tissue in the cotton pellet test *J Pharm Pharmacol* 1963, **15**, 794–797

Debray, C, R Laumonier, J Delavault & M Rousselet Les modifications de la paroi et du suc gastrique après ligature du pylore chez le rat blanc *Arch Mal Appar dig* 1950, **39**, 297–318

Delaney, J P & E Grim Experimentally induced variations in canine gastric blood flow and its distribution *Amer J Physiol* 1965, **208**, 353–358

Doll, R, J D Hill & C F Hutton Treatment of gastric ulcer with carbenoxolone sodium and œstrogens *Gut* 1965, **6**, 19–24

Domenjoz, R Comparison of therapeutically important antirheumatics on the basis of experimental results *Med Pharmacol exp* 1966, **14**, 321–331

De Elio, F J Acetylcholine antagonists A comparison of their action in different tissues *Brit J Pharmacol* 1948, **3**, 108–112

Fastier, F N, R N Speden & H Waal Prolongation of chloral hydrate sleeping time

by 5-hydroxytryptamine and by certain other drugs *Brit J Pharmacol* 1957, 12, 251–256

Forrest, A P M & C F Code The inhibiting effect of epinephrine and norepinephrine on secretion induced by histamine in separated pouches of dogs *J Pharmacol exp Ther* 1954, 110, 447–450

Franco-Browder, S , G M C. Masson & A C Cororan Induction of acute gastric lesions by histamine libarators in rats *J Allergy* 1959, 30, 1–10

Garattini, S , A Giachetti, L. Pieri & R Re Antagonists of reserpine induced eyelid ptosis *Med exp* 1960, 3, 315–320

Gati, T , F Gelencsér & L Selmeci The inhibitory effect of duodenal souring on Shay ulceration in rat. *Experientia* 1961, 17, 218–219

Ghosh, M N & H O Schild Continuous recording of acid gastric secretion in the rat *Brit J Pharmacol* 1958, 13, 54–61

Grossman, M I Some aspects of gastric secretion *Gastroenterology* 1967, 52, 882–892

Håkanson, R , B Lilja, CH Owman & S Thunell Changes in gastric secretion induced by certain amines, amine precursors and related enzyme inhibitors *Europ J Pharm* 1967, 1, 425–433

Hafter, E Die Terapie des gastro-duodenalen Ulcusschubes *Dtsch med Wschr* 1965, 90, 129–130

Halter, F , P C Ganguli, M Marshall & G P Crean Preliminary communication of the effect of gastrin II on acid secretion in conscious rats with chronic gastric fistula. *Gastroenterologia (Basel)* 1966, 106, 194–201

Hanson, H M & D A. Brodie Use of the restrained rat technique for study of the antiulcer effect of drugs *J Appl Physiol* 1960, 15, 291–294

Haroutunian, L M , H L. Segal & J J Morton The Shay rat as an assay animal for antiulcer factors II Effect of enterogastrone and miscellaneous non-specific factors *Gastroenterology* 1952, 21, 411–418

Harries, E H L The mode of action of sympathomimetic amines in inhibiting gastric secretion *J Physiol* 1957, 138, 48P–50P

Hint, H C & A. W Richter A simple intravenous infusion technique for mice *Acta pharmacol et toxicol* 1958, 14, 153–157

Hoppe, J O , E B Alexander & L C Miller Use of the trypan blue and rabbit eye tests for irritation *J Amer pharm Ass* 1950, 39, 147–151

Horakova, Z , J Metys & V Cepelak Influence de la phénylbutazone et de la cétophénylbutazone sur le tractus gastro-intestinal *Therapie*, 1965, 20, 617–630

Hunt, J N Gastric emptying and secretion in man *Physiol Rev* 1959, 39, 491–533.

Hunt J N & W R Spurrell The pattern of emptying of the human stomach. *J Physiol. (Lond)* 1951, 113, 157–168

Jacobson, E D The circulation of the stomach *Gastroenterology* 1965, 48, 85–109

Jageneau, A. & P Janssen The mydriatic activity of a series of atropine-like substances in mice *Arch int Pharmacodyn* 1956, 106, 199–206

Janssen, P A J & A H Jageneau A new series of potent analgesics *J Pharm Pharmacol* 1957, 9, 381–400

Johansson, H & G Nylander Effects of thyroxine and thiourasil treatment on gastric secretion and gastric ulcer incidence in the Shay rat. *Acta Chir Scand* 1964, 127, 527–535

Jordan, P H & R Quintana Insulin inhibition of gastrin-stimulated gastric secretion. *Gastroenterology* 1964, 47, 617–625

Jow, E., D R Webster & S C. Skoryna Effects of glucagon and insulin on gastric secretion in rats *Gastroenterology* 1960, 38, 732–739

odibenzyle relation dose-efficacité vis-à-vis de l'ulcère expérimental de contrainte *Thérapie* 1963, **18**, 373–389

Bonfils, S, J P Hardouin & Fl Delbarre Ulcération gastrique du rat blanc par administration de phénylbutazone *C R Soc Biol (Paris)* 1954, **148**, 881–884

Bonfils, S, J P Hardouin, C Richir & A Lambling Lésions gastriques du rat blanc par administration de phénylbutazone étude de quelques facteurs de "protection" *Thérapie* 1958, **13**, 490–501

Bonta, I L A study of the effect of some glucocorticoids and A C T H on artificially induced gastric ulcers of the rat *Arch int Pharmacodyn* 1961, **132**, 147–163

Bornmann, G Studie uber das sogenannte Immobilisationsulcus *Naunyn-Schmiedeberg's Arch exp Path Pharmak* 1965, **251**, 200

Brodie, B B Physico-chemical factors in drug absorption *Absorption and Distribution of Drugs* E & S Livingstone Ltd 1964 pag 16–48

Brodie, D A Ulceration of the stomach produced by restraint in rats *Gastroenterology* 1962, **43**, 107–109

Brodie, D A A comparison of anticholinergic drugs on gastric secretion, gastric emptying, and pupil diameter in the rat *Gastroenterology* 1966a, **50**, 45–50

Brodie, D A The mechanism of gastric hyperacidity produced by pylorus ligation in the rat *Amer J dig Dis* 1966b, **11**, 231–241

Brodie, D A & S K Kundrats The effect of pyrexia on rat gastric secretion *Gastroenterology* 1964, **47**, 171–178

Brodie, D A, R W Marshall & O M Moreno Effect of restraint on gastric acidity in the rat *Amer J Physiol* 1962, **202**, 812–814

Buchel, L & D Gallaire Influence de la température ambiante sur la production d'ulcères de contrainte chez le rat *C R Soc Biol (Paris)* 1966, **160**, 1817–1820

Cavallito, C J & T B O'Dell Modification of rates of gastrointestinal absorption of drugs II Quaternary ammonium salts *J Amer pharm Ass* 1958, **47**, 169–173

Cazort, R J Atropine-like action of alpha, alpha-diphenyl gamma-dimethylaminovaleramide HCl (BL 139) *J Pharmacol exp Ther* 1950, **100**, 325–332

Code, C F Histamine and gastric secretion a later look, 1955–1965 *Fed Proc* 1965, **24**, 1311–1321

Cooke, A R Role of adrenocortical steroids in the regulation of gastric secretion *Gastroenterology* 1967, **52**, 272–281

Cook, D L & R G Bianchi SC-15396 A new antiulcer compound possessing antigastrin activity *Life Sciences*, 1967, **6**, 1381–1387

Cygielman, S & J M Robson The effect of irritant substances on the deposition of granulation tissue in the cotton pellet test *J Pharm Pharmacol* 1963, **15**, 794–797

Debray, C, R Laumonier, J Delavault & M Rousselet Les modifications de la paroi et du suc gastrique après ligature du pylore chez le rat blanc *Arch Mal Appar dig* 1950, **39**, 297–318

Delaney, J P & E Grim Experimentally induced variations in canine gastric blood flow and its distribution *Amer J Physiol* 1965, **208**, 353–358

Doll, R, J D Hill & C F Hutton Treatment of gastric ulcer with carbenoxolone sodium and œstrogens *Gut* 1965, **6**, 19–24

Domenjoz, R Comparison of therapeutically important antirheumatics on the basis of experimental results *Med Pharmacol exp* 1966, **14**, 321–331

De Elio, F J Acetylcholine antagonists A comparison of their action in different tissues *Brit J Pharmacol* 1948, **3**, 108–112

Fastier, F N, R N Speden & H Waal Prolongation of chloral hydrate sleeping time

Prabhu, V G, G F Shah & S H Parikh Ulcerogenic potential of some non-steroidal anti inflammatory agents *Indian J Pharm* 1967, 29, 100–101

Pradhan, S. N & H. W Wingate Effects of some adrenergic blocking agents on gastric secretion in dogs *Arch int Pharmacodyn* 1966, 162, 303–310

Proctor, C. D, J L. Potts, L. G Ashley & B A Denefield Pilocarpine reversal of d amphetamine induced increase in mouse exploratory locomotor activity *Arch int Pharmacodyn* 1967, 167, 61–68

Ritchie, W P, J J Breen & O H Wangensteen Freezing of the intact rat stomach An indication of the biological potential of the technic *Amer J dig Dis* 1967, 12, 379–388

Robert, A & J E Nezamis Effect of cortisol on gastric ulcers of the Shay-rat *Proc Soc exp Biol (N Y)* 1958, 98, 9 12

Robert, A & J E Nezamis Polymyxin ulcers Effect of fluid intake and of corticoids *Fed. Proc* 1962, 21, 264

Robert, A, J P Phillips & J E Nezamis Production by restraint, of gastric ulcers and of hydrothorax in the rat *Gastroenterology* 1966, 51, 75–81

Rose, B & J S L Browne The distribution and rate of disappearance of intravenously injected histamine in the rat *Amer J Physiol* 1938, 124, 412–420

Rosenthale, M E. Personal communic 1966

Rossi, G, S Bonfils, F Lieffogh & A Lambling Technique nouvelle pour produire des ulcérations gastriques chez le rat blanc l'ulcère de contrainte *C R Soc Biol (Paris)* 1956, 150, 2124–2126

Roy, S K Influence of blood sugar on gastric acid secretion. *J Indian med Ass* 1964, 42, 185–186

Rubin, B, M H Malone, M H Waugh & J C. Burke Bioassay of rauwolfia roots and alkaloids *J Pharmacol exp Ther* 1957, 120, 125–136

Schanker, L S, P A. Shore, B B Brodie & C. A M Hogben Absorption of drugs from the stomach I The rat. *J Pharmacol exp Ther* 1957, 120, 528–539

Schapiro, H, A J Cummins & J Luckey Dissociation between gastric secretion and motility *Amer J dig Dis* 1965, 10, 751–757

Segal H L, L M Haroutunian & J J Morton The Shay-rat as an assay animal for anti-ulcer factors I The yield and effect of crude and purified urinary extracts from normal and abnormal individuals *Gastroenterology* 1952, 21, 400–411

Selye H Einführung in die Lehre vom Adaptionssyndrom Georg Thieme Verlag, Stuttgart 1953

Semb, L S The effects of a gastric inhibitory substance on gastric secretion in the pylorus ligated rat *Acta physiol scand* 1966, 66, 374–382

Shay, H, S A Komarov, S S Fels, D Meranze, M Gruenstein & H Siplet A simple method for the uniform production of gastric ulceration in the rat. *Gastroenterology* 1945, 5 43–61

Simler M, J Schwartz & F Schmid Effets du chlorhydrate de morphine sur la prévention de l'ulcère de contrainte chez le rat *C R Soc Biol (Paris)* 1962, 156, 1495–1497

Stephenson, R P The pharmacological properties of conessine, isoconessine and neoconessine *Brit J Pharmacol* 1948, 3, 237–245

Thompson J H & Y H Lee Determination of the dose of histamine causing maximal gastric acid secretion in the pylorus-ligated rat *Amer J dig Dis* 1967, 12, 449–455

Tonnesen, M The absorption and distribution of atropine in rats *Acta pharmacol et toxicol* 1948, 4, 367–378

Welbourn R B & C. F Code Effects of cortisone and of adrenalectomy on secretion of gastric acid and on occurrence of gastric ulceration in the pylorus ligated rat. *Gastroenterology* 1953, 23, 356–362

Kahlson, G, E Rosengren, D Svahn & R Thunberg Mobilization and formation of *histamine in the gastric mucosa as related to acid secretion* *J Physiol* 1964a, 174, 400–416

Kahlson, G, B Lilja & S E Svensson Physiological protection against gastric ulceration during pregnancy and lactation in the rat *Lancet* 1964b, II, 1269–1272

Karlmark, B & K J Obrink The antidiuretic hormone as an inhibitor of gastric secretion *Scand J Gastroent* 1967, 2, 124–128

Komarov, S A, H Shay, M Rayport & S S Fels Some observations on gastric secretion in normal rats *Gastroenterology* 1944, 3, 406–413

Kowalewski, K, W C MacKenzie, T K Shnitka & G O Bain Protective action of Pro-Banthine (SC-3171) on experimental gastric ulcers of rats *Canad med Ass J* 1954, 71, 477–482

Kubin, R H, W R Keeler & A P Klotz Influence of route of administration on therapeutic ratio of atropine in sham fed dogs *Amer J dig Dis* 1967a, 12, 363–370

Kubin, R H, W R Keeler & A P Klotz Nonparallel gastric and salivary gland inhibition produced by anticholinergic drugs in sham-fed dogs *Amer J dig Dis* 1967b, 12, 371–378

Kyle, J & R B Welbourn The effect of histamine on gastric secretion and ulceration in the pylorus ligated rat *Gastroenterology* 1956, 30, 593–597

Lambling, A, J P Hardouin, S Bonfils & R Laumonier Ulcère gastrique expérimental *Arch Mal Appar dig* 1953, 42, 417–451

Langley, P F, J D Lewis, K R L Mansford & D Smith Biochemical studies on poldine methyl methosulphate *Biochem Pharmacol* 1966, 15, 1821–1829

Leach, G D H Estimation of drug antagonisms on the isolated guinea pig vas deferens *J Pharm Pharmacol* 1956, 8, 501–503

Levine, R R Mechanisms of intestinal absorption as they relate to quaternary ammonium compounds *Arzeneimittel-Forsch* 1966, 16, 1373–1375

Lin, T M & R S Alphin Cephalic phase of gastric secretion in the rat *Amer J Physiol* 1958, 192, 23–26

Lish, P M Personal communic 1965

Lozzio, B B, L Biempica, M Royer & S Gorodisch Action of aminoguanidine on the stomach of pylorus-ligated rats *Amer J dig Dis* 1961, 6, 534–545

Madden, R J & H H Ramsburg Adrenalectomy in the Shay rat *Gastroenterology* 1951, 18, 128–134

Marner, I L Examination of gastric activity by means of dialysis in the stomach *Acta med scand* 1958, 161, 289–300

Martindale, K, G F Somers & C W M Wilson The effect of thalidomide in experimental gastric ulcers *J Pharm Pharmacol* 1960, 12 suppl 153T–158T

Menguy, R. Effects of restraint stress on gastric secretion in the rat *Amer J dig Dis* 1960, 5, 911–916

Metys, J, J Metysova & Z Horakova Influence de la phénylbutazone et de la cetophénylbutazone sur la sécrétion gastrique chez les animaux *Therapie* 1965, 20, 605–615

Miller, L C & M L Tainter Estimation of the ED_{50} and its error by means of logarithmic-probit graph paper *Proc Soc exp Biol (N Y)* 1944, 57, 261–264

Moreno, O M & D A Brodie Effects of drugs on gastric hemorrhages produced by the administration of Polymyxin B *J Pharmacol exp Ther* 1962, 135, 259–264

Newbould, B B Chemotherapy of arthritis induced in rats by mycobacterial adjuvant *Brit J Pharmacol* 1963, 21, 127–136

Parrot, J L Diminution de la perméabilité des capillaires sous l'influence de l'adrénochrome *C R Soc. Biol (Paris)* 1949, 143, 819–822

Wilhelmi, G Über die antiphlogistische Wirkung von Pyrazolen, speziell von Irgapyrin bei peroraler und parenteraler Verabreichung *Schweiz med Wschr* 1950, 80, 936–942

Wilson, T R Age and susceptibility to gastric ulceration in male and female rats *Gerontologia* 1966, 12, 226–230

Winter, C A & C C Porter Effect of alterations in side chain upon anti inflammatory and liver glycogen activities of hydrocortisone esters *J Amer pharm Ass* 1957, 46 515–519

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ANDELSBOGTRYKKERIET I ODENSE

Introduction

Bis-(p-acetoxyphenyl)-cyclohexylidene methane (F6066) and bis-(p-acetoxyphenyl)-2 methyl-cyclohexylidene methane (F6103) have been tested clinically for a number of indications, including induction of ovulation, prostatic cancer *and induced abortion Some of the animal experimental investigations which* provide the basis for these clinical trials in humans are presented in the present work

In the first section, features of the chemical development, from the first strong non steroid oestrogen, diethylstilboestrol, up to F6066 and F6103, are described The biological effects of these substances outside the antifertility field are treated on the basis of publications by the author and others and the author s unpublished results The physiology of early pregnancy and the possibility of interfering with it by using drugs are mentioned

In the experimental section of the work, the effects of F6066 and F6103 on fertility are investigated, especially the influence of these substances on existing pregnancy in mice, rats, rabbits and dogs The distribution of F6066 in the organism is described Particular attention is also paid to the possible risk of foetal malformation following administration of subabortive doses of the substances to pregnant animals

Comments on the history of non-steroid oestrogens

Even before the chemical structure of the steroid oestrogens had been finally established, in the early thirties DODDS, COOK and their collaborators synthe-
[illegible] -phenanthrenes with a weak oestrogenic (vagnotrophic)
[illegible]

[illegible]
phenylethenes were biologically active, especially with hydroxyl groups [illegible]
para position (DODDS, GOLBERG, GRUNFELD, LAWSON, SAFFER & ROBINSON 1944)

In 1937, *DODDS & LAWSON (1938) observed that impure preparations of p-*propenylphenol (anol) gave uneven biological effects when tested for their oestrogenic effect A potent impurity was later shown to be hexoestrol

During continued investigation of simple phenols, such as dihydroxystilbene, and during the attempted synthesis of various dimers of anol, DODDS, GOLBERG, LAWSON and ROBINSON (1938, 1939) prepared *stilboestrol and dienoestrol* (fig 3)

Triphenylethylene, a weak oestrogen earlier described by DODDS, was developed further by substitution with hydroxyl groups to substances with an oestrogenic effect which was pronounced, even if ten times weaker than that of stilboestrol Among others obtained was 4,4' dihydroxy α ethyl β-phenyl stilbene (DODDS *et al* 1944)

MIQUEL (1958) described an isomer of stilboestrol, interchanging one of the *ethylgroups with one of the para hydroxyphenyls* The vagnotrophic activity was 100 times weaker than that of stilboestrol

Within this series, several other substances were later described with various alkyl groups including some where the alkyl groups were connected to a saturated ring (MIQUEL, WÅHLSTAM, OLSSON & SUNDBECK 1963) Among the substances described was *bis-(p-acetoxy phenyl)-cyclohexylidene methane* (F6066) (cyclofenil), whose properties in animal experiments are described in the present publication The second substance dealt with here is bis-(p-acetoxyphenyl)-2 methyl-cyclohexylidene methane (F6103) The latter differs from F6066 by the addition of a methyl group in position 2 on the cyclohexane ring (SUNDBECK OLSSON, MIQUEL & BORRETZEN personal communication) (fig. 2)

F6066 and F6103 are white, crystalline powders, with a very low solubility in water, but soluble in ethanol and olive oil

Previous pharmacological, biochemical and clinical studies with F6066 and F6103

Some of the animal experiments reviewed in this section have been published (EINER-JENSEN 1965, 1967 a+b) A whole body radioautographic study with ^{14}C-F6066 (HANNGREN, EINER-JENSEN & ULLBERG 1965a) will be treated *on page 74* A preliminary communication has been published on the antifertility properties of F6066 (HANNGREN, EINER-JENSEN & ULLBERG 1965b) *The effects of F6103* in animal experiments have not been published previously The treatment of the previously unpublished results of experiments with F6066 and F6103 should be regarded as personal communications

The only chemical distinction between F6066 and F6103 is, as mentioned, that the latter has a methyl group situated in the 2 position on the cyclohexane ring In animal experiments, the two substances are qualitatively similar but differ quantitatively As a general principle for this quantitative difference, F6103 should be used in approximately ten times smaller doses than F6066 in order to achieve the same effect

The common definition of oestrogens, and that employed in the present work, is that they can stimulate uterine growth in immature or castrated females and bring about vaginal cornification in castrated animals To achieve these effects, F6066 and F6103 must be used in, respectively, 1000 × and 100 × greater doses than oestradiol The maximum uterine weight which can be reached with F6066 and F6103 is less than that obtained with oestradiol or stilboestrol One hundred per cent cornification of vaginal cells can be obtained with F6066 and F6103, but with doses which stimulate cornification in 50% of the animals, significantly more non cornified, nucleated cells are seen with F6066 and F6103 than with oestradiol F6066 and F6103 may thus be described as weak non-steroidal oestrogens*

In a pituitary inhibition test on immature male rats, F6066 and F6103 can reduce the weight of the testicles and ventral prostate by over 50% in one week* The seminal vesicles double in weight on this dose because they are stimulated directly by oestrogens The injection of preparations containing FSH and LH abolishes the effect of F6066 in this test

*) EINER JENSEN (1965) has published the results for F6066 marked with an asterisk on this and succeeding pages

Table 1

Pituitary inhibition test, vaginal smear test and test for uterotrophic effect of F6066, F6103, stilboestrol and oestradiol

	Effective dose for 45% decrease of		Vaginal smear from ovariecto mized rats, ED50 (3)	ED utero trophic effect mice (4)	Ratio (3)/(2)
	(1) testes	(2) v prostate			
F6103	0 02 mg	0 01 mg	0 02 mg	0 4 µg	2
F6066	0 3 mg	0 2 mg	0 8 mg	20 µg	4
Stilboestroldi propionate	>2 µg	0 7 µg	0 5 µg	0 02 µg	0 7
Oestradiolben zoate	0 4 µg	0 5 µg	0 2 µg	0 02 µg	0 4

5 rats per dose in the pituitary inhibition test, 20 ovariectomized rats per dose in the vaginal smear test and 10 immature mice per dose in the uterotrophic test were used The effective uterotrophic dose was considered to be the dose that gave the same mean uterus dry weight as 0 018 µg EB s c per animal per day for 2 days All doses stated per animal

Table 1, corresponding to table 2 of EINER-JENSEN (1965), indicates the approximate effective doses in the above-mentioned test for F6066, F6103, oestradiol benzoate (EB) and diethylstilboestrol dipropionate (DES)

F6066 and F6103 are active as oestrogens both after peroral and subcutaneous administration Their activity (determined, for example, as their uterotrophic effect) is greatest if the substances are given dissolved in oil If suspensions in gum arabic are administered to animals, the effect is more pronounced when microcrystals are used*

1 µg/animal oestradiol and stilboestrol increase the weight of *musculus levator ani* by 40% in castrated male rats F6066 does not affect the weight of this muscle*

The growth rate of the hypophysis in young, growing male rats decreases during continuous administration of 2 mg/kg F6103 for one month No tendency towards such a decrease is seen after an oestrogen equivalent dose of stilboestrol dipropionate, although the weight changes of the testes and ventral prostate are the same as in the F6103 treated animals In a corresponding experiment with growing female rats, ovariectomy or 40 µg/kg/day DES evoked an increase in hypophyseal growth rate Female rats treated with 4 mg/kg F6103 or the combination 4 mg/kg F6103 + 40 µg/kg DES had a hypophyseal weight which was lower than that of control animals The effect of F6103 in the combined treatment may be interpreted as an antioestrogenic effect. The effect on hypophyseal weight, which has not previously been published, is an im-

portant difference between the F-substances and the strong oestrogens, which will be treated in greater detail in the general discussion

F6066 seems to be a considerably stronger oestrogen for poultry than for mammals An oviduct weight of 30 mg was achieved by the injection of 0 55 mg for 5 days in one day old female chicks The corresponding dose of oestradiol benzoate was 5 × 0 025 mg An antioestrogenic effect on the chick oviduct was seen when 1 5–5 mg F6066 was given together with 0 5 mg oestradiol benzoate The degree of inhibition was between 50 and 60% with 5 mg and 25% with 1 5 mg (BARÁNY, pers comn)

PERSSON (1965 b) induced ovulation with F6066 in anovulatory women This property has been investigated in two types of non fertile experimental animals androgen sterilized rats and pseudopregnant rats

It was found that 14 out of 20 acyclic 3 month old rats (sterilized with 0 5 µg testosterone at 5 days of age) injected with 0 5 mg F6066 per animal for 3 days ovulated Ovulation was checked in every case by verifying the appearance of corpora lutea After 0 5 mg clomiphene, 80% of the animals ovulated The difference was probably not significant but the number of observations was small (Martini, personal communication)

In a similar experiment, we could confirm the changes in the vaginal smear Corpora lutea could not be demonstrated except in an untreated, semicastrated group of rats (unpublished data)

During continuous administration of 50 mg/kg of a suspension of F6066 to pseudopregnant rats, ovulation and pregnancy could be obtained in 58% of the animals, compared with 33% of untreated controls and 8% of animals treated with stilboestrol (0 04 mg/kg) and clomiphene (0 08 mg/kg) (WATNICK, NERI, TABAHNICK & PERLMAN, personal communication)

Continuous administration for up to 20 days of 4 mg/kg F6066 per os to bitches, produced ovulatory heat in all of the eight animals treated Seven of these were paired and five became pregnant (EINER-JENSEN 1967 b)

LARSSON (personal communication) treated eight mares, in whom heat failed to appear, with F6066 All eight animals came into heat after treatment and could be made pregnant

Subcutaneous administration of 5 mg F6066 per animal to four rats 4–9 days post partum had no effect on lactation, as judged by the growth of the young 25 mg/kg F6103 possibly had some effect since the young grew somewhat worse than young from female rats given olive oil alone Injection of 0 1 mg oestradiol benzoate + 1 0 mg progesterone per animal and day increases the mortality of the young from 17% for those of control mothers to 71% for those of treated mothers Any antiprolactin effect of F6066 and F6103 is therefore weak*

F6066 (30 mg in oil per animal and day given orally for two days) has no diuretic effect on male or female rats, but diuresis and sodium excretion in-

crease 2-3 days after the final dose, presumably indicating the cessation of an oestrogenic water retention*.

No anti inflammatory effect or antagonism to prednisolone acetate could be [illegible] 5 0 mg/rat F6066 in a cotton pellets test*.

[illegible]

five hours, 20 and 50 μg/kg DES has the same effect*. [illegible] an anorexigenic effect Lower doses of F6103 have not been used in this test, *although 2 5 mg/kg F6103 was observed to cause reduced food intake in a three* month toxicological experiment The mode of action has not been established, *but may depend upon inhibition of STH.*

A water soluble phosphate of F6066, F6111, was studied with respect to its effect on the prostatic secretion of sexually mature dogs Corresponding analyses were performed on dogs treated with diethylstilboestrol (DES) Daily administration of 250 mg F6111 or of 0 25 mg DES had largely the same inhibitory effect on prostatic secretion The volumes secreted diminished and both total and tartrate inhibited phosphatase activity decreased. The protein content of the secretion also diminished as well as the rate at which administered ^{65}Zn was eliminated in the prostatic secretion In the doses used, DES had a more marked effect on the volumes secreted than did F6111 (quoted from JOHNSON, NYLANDER & WIKSTROM 1966)

The acute lethal effect of F6066 and F6103 was determined after the administration of solutions in oil to mice for two days With doses of F6066 up to *1600 mg/kg, no animal died** *LD50 for F6103 was approximately 1500 mg/kg;* three out of six animals died after a dose of 1600 mg/kg. Doses greater than

Table 2

Summary of toxicological investigations with *F6066* and *F6103*

Substance	Dosage period	Dose mg/kg	Species	Number of animals per dose and sex	Form of adm	Route of adm	Number of doses per week
F6066	15 weeks	5 25, 125	rat	10	s g a	oral	6
F6066	3 months	40, 200	dog	2	tabl	oral	7
F6103	7 weeks	40 160	rat	10	oil	oral	7
F6103	3 months	2 5, 10 40	rat	5	oil	oral	7
F6103	4 months**	40	rat	5	oil	oral	7
F6103	3 months	10, 40	dog	2	gel caps	oral	7

** 3 months' dosage, 1 month's cessation before death

1600 mg/kg could not be given, since the amount of oil required was *too large*
LD50 for DES was 570 (410–780) mg/kg

Several *prolonged toxicity studies* have been carried out with F6066 and F6103 in rats and dogs (see table 2)

In these experiments growth and haematology were followed, kidney and liver function was investigated by clinical chemistry, and histopathological studies were made A dose-dependent reduction in growth rate was *shown for all* animals treated by comparison with control animals

Minor changes in liver and kidney function, which were usually reversible during the course of treatment, were demonstrated In both sexes, liver and kidney hypertrophy was observed in relation to the control animals when organ weight was calculated as a percentage of body weight, but not if the organ weight in grams was directly compared Histopathological studies performed by Dr HOJGAARD, Copenhagen, revealed no changes evoked by F6066, although the kidneys of rats treated with F6103 showed pigment in the tubuli epithelium and dose dependent, reversible changes (granular cytoplasm, slight displacement of nuclei and slight dilation of lumina) in the tubules Among the rats in the first experiment with F6103, a number of cases of nephrocalcinosis were observed this can, however, be found after other oestrogens or in female rats under special dietary conditions (COUSIN & GEARY 1966) In addition to weight changes of the gonads and secondary sex glands, a weight increase of the adrenals was demonstrated in female rats

In conclusion, no serious toxic changes have been demonstrated either with F6066 or F6103 after three months of treatment

LARSSON (1965) studied the excretion and metabolism of F6066 and the corresponding non-acetylated compound, F6060, in rats rabbits and human subjects He reported as follows ' Use was made of dihydrodiethylstilbestrol as a reference substance in the metabolic studies metabolically it proved to resemble F6060 The most reliable results were obtained with ^{14}C labelled substances Measurement of the radioactivity in the urine and faeces from rats given ^{14}C-F6060 and ^{14}C F6066 showed that the bulk of the substances was excreted in the faeces, whether the dose was given subcutaneously or perorally whereas in rabbits the bulk of the radioactivity was excreted in the urine Human urine collected for up to 6 days was found to contain 17–35% of the dose of ^{14}C F6066 given and that mainly in metabolized form The rate of excretion in all the species studied was relatively low, several % of a single dose appeared in the excreta later than on day 4 The urinary excretion products were found to consist not only of metabolized and conjugated substances, but also of highly polar compounds not extractable with ether from an acid solution In the faeces of rats and rabbits the main product was F6060 even on administration of F6066, but here, too, a small proportion of the substance had undergone metabolic conversion to polar products In bile from the

rabbit and from human subjects F6060-monoglucosiduronic acid was identified This compound was isolated in pure form from rabbit urine "

F6060 ("dihydroxy-F6066") has been shown to inhibit the *in vitro* conversion of ^{14}C pregnenolone to progesterone and other Δ^{4}-3 ketosteroids With F6060 as well as DES a 50% inhibition was noted at a concentration of 0 82–1 15 · 10^{-4} M Oestradiol-17β, however, did not show any inhibitory effect under the same conditions (LARSSON & STENSSON 1967)

F6204 ("dihydroxy-6103") is a pregnenolone oxidation inhibitor 3–5 times stronger than F6060 (LARSSON, personal communication) The dihydroxy compounds were used because F6066 and F6103 are insoluble in water

The first clinical trial with F6066 was in males with prostatic cancer after the demonstration of a relatively stronger antigonadotrophic than oestrogenic effect in animal experiments (BÁRÁNY, personal communication)

In the beginning, F6066 in a daily dosage of 600–2400 mg seemed to show a similar favourable effect on the cancer to stilboestrol, but without feminizing side effects (NYLANDER & TERNER 1964)

After an extended investigation with 90 patients and an observation time of 1–4 years, TERNER (1966) has not been able to maintain this favourable impression When the dose of F6066 was increased in cases with incipient resistance, he found feminizing side effects The number of cases exhibiting resistance to treatment also seems higher after F6066 than after DES Urinary gonadotrophin determinations hitherto carried out have not verified the pituitary inhibition effect expected from the original animal experiments, but despite this the testicles decrease in size A possible explanation of this somewhat weak effect may be poor resorption of the large doses of up to three grams per day used

PERSSON (1965a, b) performed the first clinical trials with F6066 in women, first to establish the effect on hormone excretion and later, using these results as a guide, to check the effect on menstrual disorders

PERSSON's paper is a cornerstone in the work with F6066, like that of LARSSON mentioned above, and therefore deserves quotation First "Estimations of pituitary gonadotrophins, estrogens, 17 ketosteroids and 17-hydroxy-corticosteroids have been performed in twenty two hospitalized postmenopausal patients receiving treatment with F6066

F6066 may modify the pituitary gonadotrophic function either in a stimulating or an inhibiting way as revealed by assays of "total gonadotrophins" (HPG) *in immature female mice* and LH estimations with an immunological method The outcome of treatment seems to depend on the dose administered as well as on the state of the pituitary gonadotrophic function before the therapy *There is evidence adduced to show that the effect of the drug on the HPG output may be due to change of its ' FSH/LH activity ratio '*

The urinary excretion of estradiol-17β was *rapidly nullified* during therapy at

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The physiology of early pregnancy

In the present work, the possibility of using drugs to interrupt early pregnancy in the mouse, rat, rabbit and bitch has been investigated. The experimental animals used have, almost without exception, been *mature* females. In the course of the experiment they have nearly all mated, or had the opportunity to mate, with untreated, fertile males. A short review of reproductive physiology will therefore be given in this chapter.

The primitive gonads are laid down early in foetal life, and organ differentiation and the start of sex hormone production also occur in the *foetal stage*, but administration of hormones to the mother exerts almost no effect at all on the gonads of the foetus in higher mammals (WOLFF 1962). The non gonadal genital tract can be influenced even at the foetal stage. Thus female rats with blind vaginas connecting to a common vaginal urethra are born to mothers injected with testosterone, and usually an enlarged prostate is also found.

Similar observations have been made during twin pregnancies in the cow. If the embryos are of opposite sex and *vascular connections* are established between them, the female is modified in the male direction, forming a *freemartin*. Since the structural changes are limited to the production of male sex characters in the female co-twin, it has been inferred that the gonads of the male co-twin not only produce but release sex hormones into the common blood stream at a very early stage (WILLIER 1955).

During sexual maturation of the female, a cyclic secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) begins. The secretion is to a large extent controlled by the hypothalamus, which sends "releasing factors" to the pituitary (HARRIS 1966). The ovarian hormones influence the secretion of the gonadotrophins through a feed-back system by acting on hormone sensitive centres in the hypothalamus (FLERKO 1954, FLERKO & SZENTAGOTHAI 1957). Localization of oestrogens in the pituitary has been shown by microautoradiography, and a direct effect on the pituitary is thus also a possibility (EISENFELT & AXELROD 1965).

FSH stimulates the growth of the ovarian follicles. FSH together with a slight amount of LH stimulates the follicles to produce oestrogens. Through the feed-back system between the ovaries and the hypothalamus-pituitary, the production of oestrogens causes a reduction in FSH secretion but an *increase in LH* secretion. A balance between FSH, LH and oestrogen is considered to be

all doses used The output of estrone tended to rise whereas the estriol level did not show any regular influence by therapy

Assays of 17-ketosteroids and 17-hydroxycorticosteroids did not reveal any changes of excretion during therapy "

Later, "F6066 was administered to 27 women with menstrual dysfunction with the hope of inducing regular, ovulatory menses In 19 of these patients ovulatory-like menses occurred during therapy

Ten of the patients complained of infertility of primary (6 cases) or secondary (4 cases) type Conception occurred in 6 of these cases during therapy Normal infants have been delivered in 5 and abortion occurred in one case

The correction of menstrual dysfunction by Compound F6066 is supposed to depend on the ability of the drug to modify the release of pituitary gonadotrophins Sufficient gonadotrophin producing capacity of the pituitary as well as potentially functioning ovaries are necessary pre-requisite conditions for an adequate response to the treatment "

Both ADELCREUTZ, JOHANSSON & LUUKAINEN (1967) and ARNOLD, BERGER, KELLER, RICHTER & VETTWILLER (1967) have in contrast to PERSSON proved the appearance of oestradiol in urine from F6066 treated women The discrepancy seems to depend on the similarity in physico chemical properties between oestradiol and a metabolite of F6066 ARNOLD *et al* (1967) have proved the increase in oestrone output described by PERSSON

PERSSON's work provided the stimulus for a series of other clinicians to work on the ovulation inducing effect of F6066 In a survey of European investigations LEIDEMAN (1967, pers comn) quotes 553 cases with menstrual disorders 308 of these patients were treated for sterility 80 pregnancies have been reported

A clinical investigation on F6103's antifertility effects in early pregnancies has been started Preliminary results suggest that F6103 can interfere with and eventually interrupt a normally developing pregnancy (ENGSTROM, pers comn)

In rodents, placental progesterone production never reaches the same extent as in primates, although there is some evidence for hormone production In rats, *ovariectomy* in the first two weeks of pregnancy always leads to abortion If the rats are ovariectomized in the last fourth of pregnancy and all foetuses but one are removed, the pregnancy is maintained if the placentas are left intact In mice, the placentas are retained until term only if all the foetuses are removed, so the hormone production is even less here Δ^5 3β-hydroxysteroid dehydrogenase activity with a maximum enzyme activity about 15 days after mating has been shown in the trophoblastic giant cells in both rats and mice These experiments suggest placental production of progesterone in rodents, although this has to be definitively demonstrated biochemically In the rabbit, which is a lagomorph, progesterone production by the placenta is even less pronounced, since the ovaries are essential throughout pregnancy (for references see AMOROSO & FINN 1962b, ROTHCHILD 1965 and DEANE, RUBIN, DRIKS, LOBEL & LEIPSNER 1962)

The factors which cause the corpus luteum to regress in the absence of pregnancy are several, and vary from species to species Pituitary, ovarian and uterine factors are all thought to play a partial, but unknown, role (BJORO 1966)

In rats, where prolactin has an LTH effect, administration of LH after formation of the corpora lutea curtails their productive period Many experiments in various species demonstrate the occurrence of a uterine factor, in some, but not all, of these works, it is concluded that close contact between the ovaries and uterus is important (GINTHER 1967) The problem of luteolysis is discussed in an extensive review by ROTHCHILD (1965), who also points out the importance of the self regulatory mechanisms of the ovary

Uncertainty about the nature of luteolysis urges caution in comparisons between different species and between early pregnancies and non pregnant individuals with functional corpora lutea for example women in the week *prior to* expected menstruation

In addition to progesterone, the primate placenta produces chorionic gonadotrophin (HCG) Production in women reaches a maximum in the end of the second and beginning of the third month of pregnancy, and thereafter falls to a considerably lower level which remains constant until the end of pregnancy (BORELL & RYBO 1962) HCG stimulates progesterone production by the corpus luteum of pregnancy, really an LTH effect It is not known whether HCG is of importance for the rest of the placental hormone production Hypophysectomy of rats on the twelfth day of pregnancy or later does not cause abortion, since the rat placenta secretes a similar luteotrophic hormone (ASTWOOD & GREEP 1928)

The urine of women gives a positive pregnancy reaction already 1-2 weeks after the missed period By repeated determinations in "concentrated" urine extract, pregnancy can be established even before the date of expected menstru-

necessary to evoke ovulation and the formation of corpora lutea, *although the* exact nature of this balance is unknown (BORELL & RYBO 1962)

The ovary synthetizes oestrogens and gestagens as well as producing small amounts of androgens and corticosteroids Oestrogens are also produced in other organs (adrenals, testes, placenta) in both males and females (ECKSTEIN *1962*) Progesterone is a precursor for oestrogens, corticosteroids and andro gens, and can therefore be demonstrated in all organs which produce steroids In the present work no attempt has been made to investigate the effects of *F6066* or *F6103* on ovarian androgens and corticosteroids Neither has account been taken of the extraovarian production of oestrogens and progesterone in the non pregnant female

Oestrogens are produced in the thecal cells, the granulosa cells, the corpus luteum and the interstitial cells FALCK (1959) showed with microtransplants of rat ovarian cells to the anterior chamber of the eye, that secretion of oestrogens was never recorded in transplants of the pure cell system but only in transplants containing theca interna cells or interstitial cells *combined with* granulosa cells or corpus luteum cells Some doubt about the necessity of a two cell system has, however, been raised by, for example, RYAN & SHORT (1965)

The newly formed corpus luteum is a major producer of progesterone If the animal does not become pregnant, pituitary stimulation of the corpus luteum ceases and, with it, progesterone production According to one theory, the pituitary stimulates corpus luteum formation and progesterone production in the ovary by LH secretion A second theory suggests that corpus luteum for mation is caused by LH stimulation, whilst progesterone production is caused by a special pituitary factor, the luteotrophic hormone (LTH) The occurrence of LTH is established in rodents, but in other species its presence is doubtful or can be ruled out (ROTHCHILD 1965) LTH may be identical with prolactin (HAM BURGER 1950) This would explain the persistence of corpora lutea during lactation It has even been suggested that growth hormone (STH) and LTH are identical (for references see SEGALOFF 1962)

Uncertainty about the nature of LTH and its significance for progesterone production makes it extremely difficult to discuss the effect of drugs on the luteotrophic hormone in the experiments we have performed

If the liberated egg is fertilized and implants in the uterus, the corpus luteum persists, and progesterone production from corpus luteum or placenta con tinues throughout pregnancy as a necessary condition for pregnancy main tainence The human placenta is developed and begins to produce HCG and progesterone shortly after implantation Pregnancy lasts about 267 days 1–1½ months after fertilization placental progesterone production is already so high that the ovaries can be removed without interrupting pregnancy An equivalent production of placental hormones has been demonstrated for other primates

In rodents, placental progesterone production never reaches the same extent as in primates, although there is some evidence for hormone production In rats, ovariectomy in the first two weeks of pregnancy always leads to abortion If the rats are ovariectomized in the last fourth of pregnancy and all foetuses but one are removed, the pregnancy is maintained if the placentas are left intact. In mice, the placentas are retained until term only if all the foetuses are removed, so the hormone production is even less here Δ^5 3β-hydroxysteroid dehydrogenase activity with a maximum enzyme activity about 15 days after mating has been shown in the trophoblastic giant cells in both rats and mice These experiments suggest placental production of progesterone in rodents, although this has to be definitively demonstrated biochemically In the rabbit, which is a lagomorph, progesterone production by the placenta is even less pronounced, since the ovaries are essential throughout pregnancy (for references see AMOROSO & FINN 1962b, ROTHCHILD 1965 and DEANE, RUBIN, DRIKS, LOBEL & LEIPSNER 1962)

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The ovary *synthetizes* oestrogens and gestagens as well as producing small amounts of androgens and corticosteroids Oestrogens are also produced in other organs (adrenals, testes, placenta) in both males and females (ECKSTEIN 1962) Progesterone is a precursor for oestrogens, corticosteroids and androgens, and can therefore be demonstrated in all organs which produce steroids In the present work no attempt has been made to investigate the effects of F6066 or F6103 on ovarian androgens and corticosteroids Neither has account been taken of the extraovarian production of oestrogens and progesterone in the non-pregnant female

Oestrogens are produced in the thecal cells, the granulosa cells, the corpus luteum and the interstitial cells FALCK (1959) *showed with microtransplants of rat ovarian cells to the* anterior chamber of the eye, that secretion of oestrogens was never recorded in transplants of the pure cell system but only in transplants containing theca interna cells or interstitial cells *combined with* granulosa cells or corpus luteum cells Some doubt about the necessity of a two cell system has, however, been raised by, for example, RYAN & SHORT (1965)

The newly formed corpus luteum is a major producer of progesterone If the animal does not become pregnant, pituitary stimulation of the corpus luteum ceases and, with it, progesterone production According to one theory, the pituitary stimulates corpus luteum formation and progesterone production in the ovary by LH secretion A second theory suggests that corpus luteum formation is caused by LH stimulation, whilst progesterone production is caused by a special pituitary factor, the luteotrophic hormone (LTH) The occurrence of LTH is established in rodents, but in other species its presence is doubtful or can be ruled out (ROTHCHILD 1965) LTH may be identical with prolactin (HAMBURGER 1950) This would explain the persistence of corpora lutea during lactation It has even been suggested that growth hormone (STH) and LTH are identical (for references see SEGALOFF 1962)

Uncertainty about the nature of LTH and its significance for progesterone production makes it extremely difficult to discuss the effect of drugs on the luteotrophic hormone in the experiments we have performed

If the liberated egg is fertilized and implants in the uterus, the corpus luteum persists, and progesterone production from corpus luteum or placenta continues throughout pregnancy as a necessary condition for pregnancy maintainence The human placenta is developed and begins to produce HCG and progesterone shortly after implantation Pregnancy lasts about 267 days 1–1½ months after fertilization placental progesterone production is already so high that the ovaries can be removed without interrupting pregnancy An equivalent production of placental hormones has been demonstrated for other primates

In rodents, placental progesterone production never reaches the same extent as in primates, although there is some evidence for hormone production In rats, ovariectomy in the first two weeks of pregnancy always leads to abortion If the rats are ovariectomized in the last fourth of pregnancy and all foetuses but one are removed, the pregnancy is maintained if the placentas are left intact. In mice, the placentas are retained until term only if all the foetuses are removed, so the hormone production is even less here Δ^5 3β hydroxysteroid dehydrogenase activity with a maximum enzyme activity about 15 days after mating has been shown in the trophoblastic giant cells in both rats and mice These experiments suggest placental production of progesterone in rodents, although this has to be definitively demonstrated biochemically In the rabbit, which is a lagomorph, progesterone production by the placenta is even less pronounced, since the ovaries are essential throughout pregnancy (for references see AMOROSO & FINN 1962b, ROTHCHILD 1965 and DEANE, RUBIN, DRIKS, LOBEL & LEIPSNER 1962)

The factors which cause the corpus luteum to regress in the absence of pregnancy are several, and vary from species to species Pituitary, ovarian and uterine factors are all thought to play a partial, but unknown, role (BJORO 1966)

In rats, where prolactin has an LTH effect, administration of LH after formation of the corpora lutea curtails their productive period Many experiments in various species demonstrate the occurrence of a uterine factor, in some, but not all, of these works, it is concluded that close contact between the ovaries and uterus is important (GINTHER 1967) The problem of luteolysis is discussed in an extensive review by ROTHCHILD (1965), who also points out the importance of the self regulatory mechanisms of the ovary

Uncertainty about the nature of luteolysis urges caution in comparisons between different species and between early pregnancies and non pregnant individuals with functional corpora lutea for example women in the week prior to expected menstruation

In addition to progesterone, the primate placenta produces chorionic gonadotrophin (HCG) Production in women reaches a maximum in the end of the second and beginning of the third month of pregnancy, and thereafter falls to a considerably lower level which remains constant until the end of pregnancy (BORELL & RYBO 1962) HCG stimulates progesterone production by the corpus luteum of pregnancy, really an LTH effect It is not known whether HCG is of importance for the rest of the placental hormone production Hypophysectomy of rats on the twelfth day of pregnancy or later does not cause abortion, since the rat placenta secretes a similar luteotrophic hormone (ASTWOOD & GREEP 1928)

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The urine of women gives a positive pregnancy reaction already 1–2 weeks after the missed period By repeated determinations in "concentrated" urine extract pregnancy can be established even before the date of expected menstru-

ation This entails a combined immunological determination of LH and HCG (LEBECK, personal communication)

Ovulation and pseudopregnancy can be obtained after pairing with vasectomized males and by electrical stimulation of the cervix in mice and rats (FINN 1965, VELARDO & HISAW 1951) Mechanical or chemical irritation of the endometrium evokes an optimal decidual reaction if the irritation occurs at the time of normal implantation, but the unfertilized egg degenerates (SHELESNYAK 1960) Gonadectomized animals can be used to determine which ovarian hormones must be administered to attain a decidual reaction Progesterone is essential, but a small amount of oestrogen is also required, especially 3–4 days after stimulation The decidual reaction fails to appear if too little or too much oestrogen is given (EMMENS 1964)

Interruption of pregnancy with drugs

The fertility of female individuals can already be influenced by drugs in foetal life *Masculinization of the female foetus* is seen after treatment with certain synthetic gestagens during late pregnancy in rodents (KINCL & DORFMAN 1962) and women (JOHNSEN 1966) In animals, this is followed by reduced fertility in adult life, the effect on fertility in women is so far an open question

Reversible, pharmacological contraceptive methods are available for mature animals, for example, administration of a combination of oestrogen and gestagen to mature females The oestrogen gestagen combination finds practical application in veterinary practice, especially for synchronization of heat, which occurs following withdrawal in the sheep and cow (RUDEL & KINCL 1966)

In women, ovulation is suppressed if the combination (the ' P-pill") is given for 21-22 day periods with 7 days intermission Withdrawal bleeding occurs about 24 days from the beginning of each period of treatment (PINCUS 1965) Today 20-30 million women use the combination for contraceptive purposes The mechanism of action is incompletely understood, but several factors beside inhibition of ovulation may be important changes in ovarian biogenesis, tubal transport, implantation, cervical mucus (for further details see DICZFALUSY 1967)

Apart from the pharmacological contraceptive methods, there is a series of techniques based on hindrance of the sperm from reaching the egg They are based on an impenetrable membrane and/or spermicides or on the so-called safe period ' (BONNEVIER, FOG & RIIS 1966)

Insertion of intrauterine devices (IUD) has been used by the Arabs for thousands of years as a traditional method for preventing undesirable pregnancies in camels

In the 1920s, GRÄFENBERG inserted rings of silk or silver *in the uteri of* women in an attempt at contraception, and found the method usable (SOUTHAM 1965) It gained a bad reputation some years later because other clinicians found a high frequency of side-effects

There was a renaissance of the method after 1959 (OPPENHEIMER 1959, ISHIHAMA 1959) and intrauterine devices of plastic material are now used by hundreds of thousands of women

The action of the IUD varies from species to species (ECKSTEIN 1967) The exact mechanism in women is unknown, but does not involve inhibition of ovulation An alteration of tubal transport or an unphysiological milieu in the uterus is believed to be the reason why the women do not become pregnant Speculation on the luteolytic factor (see page 15) should also be included in any discussion of the mechanism of action

Existing contraceptive methods have one character in common they all require planning before coitus Even the IUD requires planning, although it should presumably be considered as a postconceptional method, since it is the fertilized egg which is not offered the necessary conditions for survival

No postconceptional methods based on drugs are used today in women on any great scale

Oestradiol and stilboestrol are used, more or less officially, to interrupt pregnancy in women, especially after rape They are probably effective, but there are few reports on the method in the literature

MORRIS & WAGENEN (1966) gave 5–50 mg stilboestrol or 0 5 mg ethinyloestradiol to an unspecified number of women, either rape cases or courageous volunteers, 4–6 days after coitus No pregnancies occurred in what the authors claim is the first published work on humans with postcoital contraceptive sub stances Administration of 5 mg ethinyloestradiol for 7 days 1–2 weeks after implantation does not induce abortion (DICZFALUSY 1967)

Given later in pregnancy, the effect of a strong oestrogen, diethylstilboestrol propionate, seems to be pregnancy maintenance SMITH & SMITH (1949) treated 152 primigravida, having no signs of pregnancy complications with DES from the 12th to the 35th weeks of pregnancy In the twelfth week the dose was 12 5 mg, from the fourteenth week 25 mg, rising steadily to 125 mg in the last four weeks The frequency of complications was low compared with that in an equivalent group of 283 untreated pregnancies In a similar series 235 women treated with almost the same regime were compared with 272 untreated cases The results suggested that DES in these large doses has a pregnancy maintaining effect with a reduced risk of complications

KARNAKY (1950) used much larger doses of stilboestrol to treat habitual abortion 25 mg of micronized stilboestrol were given as early as the third to seventh day after the missed period and thereafter ¼ of this dose for 3½ months In cases of threatened abortion, 100 mg were given every 15 minutes until the symptoms disappeared, and thereafter 75 mg daily for one week

Investigations by SHELESNYAK (1960) have established that a single adminis tration of ergocornine to rats during the prenidation stage of pregnancy or comparably the first week of pseudopregnancy, terminates pregnancy or pseudopregnancy Later SHELESNYAK proposed on the basis of available data a relationship between ergocornine and progester(
the theory in human volunteers

Pregnanediol determinations were made in five normally menstruating women treated after ovulation with 2 mg ergocornine A marked fall in pregnanediol content was established in urine collected during the first 24 hours after treatment The authors are of the opinion that this suggests an inhibition of the pregnenolone progesterone conversion through an inhibition of 3β-hydroxysteroid dehydrogenase (SHELESNYAK, LUNENFELD & HORNIG 1963) This biochemical action is reminiscent of the effect of F6060 and F6204 described by LARSSON (see page 11)

It was shown later that in rabbits treated on day 3–4 of gestation, 1 5–2 5 mg/kg ergocornine methanesulphonate showed no demonstrable effect and 4–6 mg/kg caused death of the mothers (MORRIS, WAGENEN, HURTEAU, JOHNSTON & CARLSEN 1967)

In newer publications (LINDNER & SHELESNYAK 1967 and LINDNER, LUNENFELD & SHELESNYAK 1967) the direct inhibiting action of ergocornine on ovarian 3β-hydroxysteroid dehydrogenase could not be confirmed and no changes in urinary pregnanediol excretion in post ovulatory ergocornine treated women was seen

A series of alkaloids, other than ergocornine, and antimetabolites have been shown to interrupt pregnancy in rat experiments Some of these have shown no effect in experiments with rabbits or monkeys (MORRIS *et al* 1967) A review of the results from this and other works is beyond the scope of the presentation given here

A number of plants contain substances with oestrogenic activity Such substances are found, for example, in hops (and therefore in beer) and in certain species of clover This group must also be considered as beyond the scope of this work although in certain cases an influence on fertility has been observed

Experiments with pregnancy interrupting substances in human clinical practice involve a number of ethical and legal problems which, in many details, are equivalent to those concerning euthanasia. The sparsity of information from clinical practice therefore contrasts markedly with the number of substances found active in animal experiments It seems that the contrast is here much greater than in many other developing fields of pharmacology

In animal experimental work with antifertility postconceptional drugs it is reasonable to distinguish between those which affect the soujourn of the free egg in the tubes or uterus and those which affect the implant egg

The most commonly described pregnancy interrupting effect is that during the time before implantation or the period of decidual development An unspecific action on these complicated mechanisms seems relatively easy to obtain On the other hand, late interruption of pregnancy with hormonally active substances has less frequently been described

Among all types of substances examined for pregnancy interrupting properties, the oestrogens are the most active and the most thoroughly investigated

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Six oestrogens were compared as to their ability to inhibit the decidual growth induced by a standard dose of progesterone in pseudopregnant rats castrated on day 5 after electrical stimulation of the vagina Oestradiol-17β was by far the most active inhibitor The other oestrogens, listed in order of their effectiveness, were oestradiol benzoate, diethylstilboestrol, oestrone, oestriol, and equilenin, the latter two being 200–400 times less active than oestradiol (VELARDO & HISAW 1951)

In rabbits larger doses of oestrogens will provoke placental separation and death of the foetus if given after implantation Microscopic sections show changes at the site of implantation with necrosis, loss of decidua, and vacuolization and degeneration of the endometrium In the macaque, the oral or intramuscular administration of stilboestrol or oestradiol dipropionate after implantation (on days 18 to 67) appeared to have little effect on the development of the foetus Of 20 animals so treated, 3 aborted, an incidence of 15 per cent This does not vary significantly from the 10 per cent expected abortion rate in the colony No pregnancies occurred in a total of 28 monkeys if stilboestrol (1 25 mg), oestradiol (10 mg) or ORF 3858 (10 mg) (see page 23) was given orally for 6 days following positive mating (MORRIS & WAGENEN 1966)

The authors of the above mentioned works are agreed that oestrogens can interrupt early pregnancies, presumably through a shift in the oestrogen-gestagen balance, although there is some disagreement as to the exact mode of action

Several substances which are chemically related to F6066 and F6103 have been described as antifertility drugs They can be grouped as triphenylethylenes (triphenylethenes), sometimes with the steric configuration stabilized by a ring structure It must be mentioned that in some of the publications cited here the chemistry is the main point and the pharmacology an appendix

The first group includes 1 (p-β diethylaminoethoxyphenyl)-1,2-diphenyl 2-chloroethylene (clomiphene) and the *trans*-isomer of 1-(p-β-dimethylaminoethoxyphenyl)-1-2 diphenylbut-1-ene (ICI 46 474) The formulae are shown in fig 1 a + b

Clomiphene is *trans cis* mixture, whereas the *cis*- and *trans* isomers of the latter substance have been prepared as isolated forms ICI 47 699, the *cis*-isomer of ICI 46 474, behaves in all respects as a conventional, orally active oestrogen such as dienoestrol

Both clomiphene (HOLTKAMP, GRESLIN, ROOT & LERNER 1960) and ICI 46 474 (HARPER & WALPOLE 1966, 1967) are weak, atypical oestrogens with pronounced antioestrogenic effects The antifertility effect in rats during the first day after mating may depend upon an oestrogenic effect on tubal transport The effect on days 4 and 5 may possibly depend on the substances' anti-

Here, as in other cases, it is impossible to precisely define a drug's effects simply by describing it as an oestrogen Ideally one should define a substance's effects in specified tests in specified species, but nevertheless for practical reasons, it is often necessary to use the term oestrogen In the present work, all substances which can evoke uterine hypertrophy in immature animals and vaginal cornification in ovariectomized animals are regarded as oestrogens

Another definition necessary for practical reasons is the term "conventional oestrogens" In this work, this term covers the oestrogens most commonly used in experimental endocrinology, such as oestradiol, oestradiol benzoate and DES The term should be understood as a contrast to the newer non steroid oestrogens, often weaker as reckoned per milligramme, which have not yet attained a definitive pharmacological position

Oestrogenic activity measured as uterine growth in immature animals and as changes in vaginal cytology in castrated animals – the classical definitions – need not be correlated Even the species of animal must be specified, there may be different relations between the growth of the oviduct in chickens and of the uterus in mice (BÁRANY, pers comn) The *trans*-isomer of a triphenylethylene has the greater oestrogenic activity in mice, whilst the *cis*-isomer has the greater activity in rats (HARPER & WALPOLE 1966) The problem seems to be greatest for the weak oestrogens Here there is an additional complication, since weak oestrogens are often antioestrogenic against strong oestrogens, and therefore may even be thought to affect the implantation mechanism through this property

The oestrogenic effects (vaginotrophic and uterotrophic) of chemically related steroids need not run parallel with their antifertility effects (BENNET, KENDLE, VALLANCE & VICKERY 1966)

As can be seen from the following examples, the antifertility effect of high doses of oestrogens is well established When pregnant mice were given oestradiol-17β, 0 15 µg s c , on days 0, 1 and 2 (mating = day 0), the mean number of young per mother fell from 6 5 to 2 5 and the mean length of pregnancy was increased by 1–2 days When the same dose was given on days 3, 4 and 5, no young were delivered (MARTIN 1963) The author suggests that in the first case there was interference with tubal function, causing tube-locking of the developing ovum, whereas treatment on days 3, 4 and 5 interfered with uterine function, preventing implantation Simultaneous administration of progesterone did not appear to give protection against the oestrogen's pregnancy interrupting effect, although other changes evoked in the uterus and vagina by the oestrogen were counteracted

Complete inhibition of implantation *in the rat may be accomplished* by a single dose of 20 µg oestrone per rat, which causes premature passage of tubal ova into the uterus (BANIK & PINCUS 1964)

Administration of progesterone to rabbits before ovulation or oestrogen after

ovulation speeds the transport of eggs and causes their degeneration or expulsion from the uterus (CHANG 1966)

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Clomiphene is *trans-cis* mixture, whereas the *cis*- and *trans* isomers of the latter substance have been prepared as isolated forms ICI 47 699, the *cis*-isomer of ICI 46 474, behaves in all respects as a conventional, orally active oestrogen such as dienoestrol

Both clomiphene (HOLTKAMP, GRESLIN, ROOT & LERNER 1960) and ICI 46 474 (HARPER & WALPOLE 1966, 1967) are weak, atypical oestrogens with pronounced antioestrogenic effects The antifertility effect in rats during the first day after mating may depend upon an oestrogenic effect on tubal transport. The effect on days 4 and 5 may possibly depend on the substances' anti-

Here, as in other cases, it is impossible to precisely define a drug's effects simply by describing it as an oestrogen. Ideally one should define a substance's effects in specified tests in specified species, but nevertheless for practical reasons, it is often necessary to use the term oestrogen. In the present work, all substances which can evoke uterine hypertrophy in immature animals and vaginal cornification in ovariectomized animals are regarded as oestrogens.

Another definition necessary for practical reasons is the term "conventional oestrogens". In this work, this term covers the oestrogens most commonly used in experimental endocrinology, such as oestradiol, oestradiol benzoate and DES. The term should be understood as a contrast to the newer non steroid oestrogens, often weaker as reckoned per milligramme, which have not yet attained a definitive pharmacological position.

Oestrogenic activity measured as uterine growth in immature animals and as changes in vaginal cytology in castrated animals – the classical definitions – need not be correlated. Even the species of animal must be specified, there may be different relations between the growth of the oviduct in chickens and of the uterus in mice (BARÁNY, pers. comm.). The *trans*-isomer of a triphenylethylene has the greater oestrogenic activity in mice, whilst the *cis*-isomer has the greater activity in rats (HARPER & WALPOLE 1966). The problem seems to be greatest for the weak oestrogens. Here there is an additional complication, since weak oestrogens are often antioestrogenic against strong oestrogens, and therefore may even be thought to affect the implantation mechanism through this property.

The oestrogenic effects (vaginotrophic and uterotrophic) of chemically related steroids need not run parallel with their antifertility effects (BENNET, KENDLE, VALLANCE & VICKERY 1966).

As can be seen from the following examples, the antifertility effect of high doses of oestrogens is well established. When pregnant mice were given oestradiol 17β, 0.15 μg s.c., on days 0, 1 and 2 (mating = day 0), the mean number of young per mother fell from 6.5 to 2.5 and the mean length of pregnancy was increased by 1–2 days. When the same dose was given on days 3, 4 and 5, no young were delivered (MARTIN 1963). The author suggests that in the first case there was interference with tubal function, causing tube-locking of the developing ovum, whereas treatment on days 3, 4 and 5 interfered with uterine function preventing implantation. Simultaneous administration of progesterone did not appear to give protection against the oestrogen's pregnancy interrupting effect, although other changes evoked in the uterus and vagina by the oestrogen were counteracted.

Complete inhibition of implantation in the rat may be accomplished by a single dose of 20 μg oestrone per rat, which causes premature passage of tubal ova into the uterus (BANIK & PINCUS 1964).

Administration of progesterone to rabbits before ovulation or oestrogen after

ovulation speeds the transport of eggs and causes their degeneration or expulsion from the uterus (CHANG 1966)

Six oestrogens were compared as to their ability to inhibit the decidual growth induced by a standard dose of progesterone in pseudopregnant rats castrated on day 5 after electrical stimulation of the vagina Oestradiol-17β was by far the most active inhibitor The other oestrogens, listed in order of their effectiveness, were oestradiol benzoate, diethylstilboestrol, oestrone, oestriol, and equilenin, the latter two being 200–400 times less active than oestradiol (VELARDO & HISAW 1951)

In rabbits larger doses of oestrogens will provoke placental separation and death of the foetus if given after implantation Microscopic sections show changes at the site of implantation with necrosis, loss of decidua, and vacuolization and degeneration of the endometrium In the macaque, the oral or intramuscular administration of stilboestrol or oestradiol dipropionate after implantation (on days 18 to 67) appeared to have little effect on the development of the foetus Of 20 animals so treated, 3 aborted, an incidence of 15 per cent This does not vary significantly from the 10 per cent expected abortion rate in the colony No pregnancies occurred in a total of 28 monkeys if stilboestrol (1–25 mg), oestradiol (10 mg) or ORF 3858 (10 mg) (see page 23) was given orally for 6 days following positive mating (MORRIS & WAGENEN 1966)

The authors of the above mentioned works are agreed that oestrogens can interrupt early pregnancies, presumably through a shift in the oestrogen-gestagen balance, although there is some disagreement as to the exact mode of action

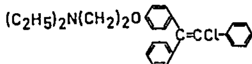
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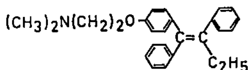
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a



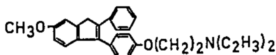
Clomiphene

b



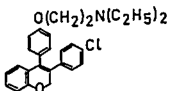
ICI 46 474

c



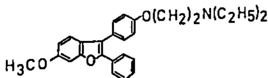
U-11 555 A

d



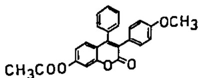
"Compound VII"

e



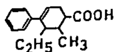
"Compound IV"

f



"Compound XIII"

g



ORF 3858

Fig. 1

oestrogenic properties, which disturb the delicate balance between oestrogens and gestagens necessary for implantation The minimal effective dose (MED) of ICI 46 474 for abortion in rats is 0 1–0 4 mg/kg on days 1–4 of pregnancy, but on day 5, 20 mg/kg is ineffective This suggests that the oestrogenic properties are not critical for the effect In HARPER & WALPOLE's experiments with dienoestrol, MED was found to be between 0 05 and 0 15 mg/kg on days 1–4 and 0 4 mg/kg on day 5 A sharp division between days 4 and 5 is not found in experiments with clomiphene (HOLTKAMP *et al* 1960)

ICI 46 474 (HARPER & WALPOLE, pers comm) and clomiphene (STAPLES 1966) exert their abortive effect via the mother, since blastocysts transplanted from treated mothers to an untreated pseudopregnant uterus develop normally

U-11555A, 2-[p-(6 methoxy-2 phenylinden-3-yl)-phenoxy] triethylamine is an example of the triphenylethenes with a fixed configuration (fig 1c)

0 5 mg/kg U-11555A given by mouth to rats, from pro-oestrus to day 6, was 100% effective in preventing pregnancy A single dose of 2 5 mg/kg was also 100% effective when given on day 4, but ineffective when 25 mg/kg was given on day 5 U 11555A is uterotrophic in castrated female rats but also acts as an antioestrogen against injected oestradiol (DUNCAN, STUCKI, LYSTER & LEDNICKER 1962) Thus the properties of U-11555A are reminiscent of those of ICI 46 474

U-11555A, like a number of the substances described here, has been selected for special investigation from a series of chemically related compounds (LEDNICKER BABBOCK, LYSTER, STUCKI & DUNCAN 1961)

[illegible]

(BENCZE, CARNEY, BARSKY, RENZI, DORFMAN & STEVEN 1965)

2,3-diphenylbenzofuranes, especially compound IV, fig 1e, also prevent the continuance of pregnancy in rats when given continually from day 0 to day 4 (GROVER, CHAWLA, ANAND, KAMBOJ & KAR 1965)

The abortive action of both the latter groups of substances is reported to diminish rapidly after the first four days of rat pregnancy

3,4-diphenylcoumarins e g compound XIII (fig 1f) have been tested for antifertility properties The majority of the neutral coumarins showed some potency, which may be based on their oestrogenic activity (LEDNICKER, LYSTER & DUNCAN 1965)

One weak oestrogen, with a chemical structure quite unlike those hitherto mentioned, deserves to be noted since it has been tested on monkeys 2 methyl-3-ethyl-4-phenyl-Δ^4-cyclohexanecarbonic acid, ORF 3858 (fig 1g), is described as nontoxic, nonteratogenic and 100% effective as an antifertility agent in rodents, with an "all or none" antifertility response in the rabbit. When macaques were given 10 mg doses on the first 6 days after mating, no preg-

nancies occurred ORF 3858 is thus effective in primates during a short period after conception The mechanism of action may very well be similar to that suggested by MORRIS & WAGENEN for stilboestrol and ethynyloestradiol (MORRIS & WAGENEN 1966, MORRIS, WAGENEN, MCCANN & JACOB 1967)

The aim of the present investigation has been to examine the effects of F6066 and F6103 upon physiological mechanisms which are of consequence in the commencement and course of pregnancy in the mouse, rat, rabbit and dog F6066 and F6103 are both nonsteroidal, weak oestrogens, and since it is known that conventional oestrogens have antifertility properties, reference substances of this type have been included in the investigation in order to determine whether eventual antifertility effects of F6066 and F6103 depend upon properties other than the conventionally defined oestrogen effect

In the account of the experiments carried out, the treatment of each method will include an introduction, a description of the method, the results and a commentary In the general discussion which concludes the work, the results of the various experiment are discussed in relation to each other and to the information in the literature

General experimental conditions

The experiments have been performed at the Pharmacological Department of Ferrosan Ltd, Malmo, although the autoradiographical work was carried out at the Department of Pharmacology, Royal Veterinary College, Stockholm

Albino mice were used *from a closed colony maintained* by a commercial breeder, Dr FRIIS, at Laven, Denmark, this colony originates from the Naval Medical Research Institute, USA An exception was in the work with whole animal autoradiography, where N MRI mice from Anticimex, Stockholm, were used The rats were from Ferrosan's 20 year-old closed colony of Sprague-Dawley descent The rabbits were purchased from a commercial breeder, Dr MOLLEGARD, at Havdrup, Denmark, they were raised in a closed colony and were of the white Danish Land race The dogs used were Beagles from Ferrosan s own breeding stock

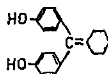
All animals had free access to water The mice and rats were fed Teknosan breeding pellets The rabbits in the anti Clauberg experiments were fed Fors rabbit pellets and those in the abortion-teratological experiments were given Teknosan rabbit pellets In addition, the rabbits received hay and carrots The animals rooms were ventilated with humidified air (50% relative humidity) which was changed seven times per hour The temperature in the mouse and rat rooms was 21 23°C and in the rabbit rooms 20°C The illumination in the animal rooms varied seasonally, but with a minimum of 10 hours per day The research laboratories have been described previously (EINER-JENSEN 1966) The dogs were kept at a kennel outside Malmo and were fed with offals

F6066, F6103 and the lipoid soluble reference substances, such as stilboestrol dipropionate, were dissolved before use in olive oil (pharmaceutical grade) containing 4% by volume of absolute alcohol The substances were weighed on an analytical balance to an accuracy of ±0 03 mg, the amount weighed always *exceeding 10 mg The solutions* were prepared in graduated flasks Where heating was necessary, the volume of oil was adjusted after cooling to room temperature Serial dilution was performed by the *transfer of solutions in oil* with disposable plastic syringes

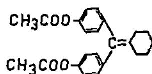
Various dilutions of oestradiol benzoate solution were prepared from a 1 mg/ml stock solution in absolute alcohol, which was renewed once yearly The

Fig 2 The following Ferrosan substances were used in the experiments or are otherwise mentioned

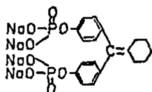
F6060 Bis-(p hydroxyphenyl)cyclohexylidenemethane



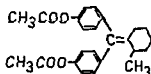
F6066 Bis (p-acetoxyphenyl)cyclohexylidenemethane



F6111 Tetrasodium bis-(p hydroxyphenyl)-cyclohexylidene-methane-diphosphate Contains 5% dimer and traces of the trimer



F6103 Bis-(p-acetoxyphenyl)-2 methyl cyclohexylidene methane



F6204 Bis (p hydroxyphenyl)-2-methyl cyclohexylidene-methane

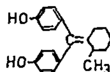


Table 3

The other substances used were

17β-oestradiol benzoate, Mann Research Laboratories, New York, USA (EB)

1,3,5,(10)-oestratrien-17α-ethynyl 3,17 diol, Mann Research Laboratories (Ethynyloestradiol)

Diethylstilboestrol dipropionate, L Light & Co , Ltd , Colnbrook England (DES)

Dienoestrol, Mann Research Laboratories From this, by acetylation with acetic anhydride, *dienoestrol diacetate* has been prepared melting point 117–119 C (*In the present work the diacetate is referred to as dienoestrol*)

Progesterone, ACO Sweden (Progesterone) 25 mg/ml

5-pregnen 3β-ol 20 one, Mann Research Laboratories (Pregnenolone)

Gonadex, Leo Ltd , Sweden According to the manufacturers, an HCG preparation from the urine of pregnant women

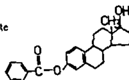
Prolactin, Ferring Ltd , Sweden According to the manufacturers, a *highly purified* prolactin preparation for research purposes

Trypan blue, Trypan blau, Standard Fluka, Switzerland

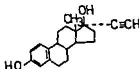
Alizarin, Alizarin pro analysi, Merck, Germany

Fig 3 The following oestrogens were used as reference substances

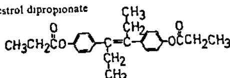
Oestradiol monobenzoate



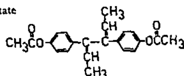
Ethynyl oestradiol



Diethylstilboestrol dipropionate



Dienoestrol diacetate



biological effect of the new solution was tested against that of the old one in a vaginal cornification test in rats

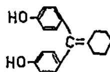
The doses used were calculated in mg per kg This method was preferred to administration in mg per animal since i a age and body weight is correlated During more prolonged experiments, the animals were weighed once a week and the doses adjusted to account for weight changes

For injection and tube feeding, tuberculin glass syringes were used, graduated to an accuracy of 0 01 ml In most experiments, 1 ml/kg olive oil was given to rats and rabbits, and 10 ml/kg to mice Since the maximum solubility of F6066 in olive oil is about 10–15 mg/ml, it was occasionally necessary to increase the volume of olive oil to 2-5 ml/kg for rats and rabbits

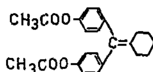
Organs of less than one gram were weighed on an analytical balance to ±0 03 mg, and those between one and ten grams on an analytical balance or a balance with 10 mg accuracy Animals were weighed to an accuracy of at

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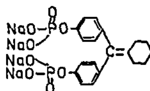
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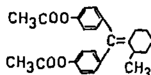
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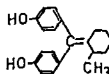


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The effect on fertility of daily administration of F6066, F6103 or DES to female rats

Introduction

The method used is an unspecific test for the antifertility effects of drugs in female rats It is based upon daily administration of the substance to the female before and during her contact with the male The advantage of the method is that it reveals eventual antifertility properties irrespective of the mechanism involved The method can also reveal the degree and speed of reversibility of such effects if the male and female are kept in the same cage after cessation of treatment, and in certain cases may also reveal teratogenic properties

Method

F6066, in doses of 0 5, 2 5 or 12 5 mg/kg, or olive oil alone, 1 ml/kg, was given daily (seven times a week) by subcutaneous injection for four weeks to three month old female rats Each group consisted of 10 females, two animals sharing each cage After the first week of injections, a male was placed in each cage and the females were given injections for a further three weeks

Two weeks after the course of injections ceased, the males were removed and the females observed for a further three weeks for signs of pregnancy Pregnant animals were placed in separate cages and the numbers of dead and living young reckoned at birth The probable date of mating was calculated as the date of birth minus 21 days Animals which did not deliver were sacrified and their uteri examined

Equivalent experiments were performed with F6103, 0 05, 0 25 and 1 25 mg/kg, and DES, 0 5, 2 5 and 12 5 μg/kg

Results

All three substances, F6066, F6103 and DES, showed dose dependent anti fertility properties in this test. The group given 0 5 μg/kg DES s c daily showed no sign of decreased fertility, in all other dosage groups fertility was affected to a greater or lesser extent

All control animals in the three series became pregnant whilst receiving oil injections

least two figures Weights of both organs and animals were afterwards approximated to two figures

Extensive statistical treatment of the material is not suitable, owing to the limited number of animals per dose and the relatively large dosage intervals used Calculations to estimate ED50 or ratios to estimate correlation between effects for different substances are therefore given only to within one figure

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Two weeks after the course of injections ceased, the males were removed and the females observed for a further three weeks for signs of pregnancy Pregnant animals were placed in separate cages and the numbers of dead and living young reckoned at birth The probable date of mating was calculated as the date of birth minus 21 days Animals which did not deliver were sacrified and their uteri examined

Equivalent experiments were performed with F6103, 0 05, 0 25 and 1 25 mg/kg and DES, 0 5, 2 5 and 12 5 μg/kg

Results

All three substances, F6066, F6103 and DES, showed dose dependent antifertility properties in this test The group given 0 5 μg/kg DES s c daily showed no sign of decreased fertility, in all other dosage groups fertility was affected to a greater or lesser extent

All control animals in the three series became pregnant whilst receiving oil injections

least two figures Weights of both organs and animals were afterwards approximated to two figures

Extensive statistical treatment of the material is not suitable, owing to the limited number of animals per dose and the relatively large dosage intervals used Calculations to estimate ED50 or ratios to estimate correlation between effects for different substances are therefore given only to within one figure

The preparation of ^{14}C F6066 and ^{14}C F6060 for intravenous administration is dealt with in the section on autoradiography

The effect on fertility of daily administration of F6066, F6103 or DES to female rats

Introduction

The method used is an unspecific test for the antifertility effects of drugs in female rats It is based upon daily administration of the substance to the female before and during her contact with the male The advantage of the method is that it reveals eventual antifertility properties irrespective of the mechanism involved The method can also reveal the degree and speed of reversibility of such effects if the male and female are kept in the same cage after cessation of treatment, and in certain cases may also reveal teratogenic properties

Method

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Equivalent experiments were performed with F6103, 0 05, 0 25 and 1 25 mg/kg and DES 0 5, 2 5 and 12 5 µg/kg

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treatment with 2 5 and 12 5 mg/kg F6066, where no animals became normal pregnant during treatment, 7 and 5 animals, respectively, became pregnant in the weeks following treatment

Comments

The experiments demonstrate that F6066 and F6103 act as antifertility substances when injected daily in female rats for one week before and during contact with male rats

DES has been used in the present experiments since it is known that conventional oestrogens (defined on page 20) can evoke sterility when continuously administered

WATNICY, GIBSON, VINEGRA & TOLKSDORF (1964) gave ethynyloestradiol by gavage once a day for 11 or 45 days to female mature rats housed with males The minimum protective dose (MPD_{100}) proved to be approximately 0 05 mg/kg/day for both the 11 and 45 days trial This dose, which was totally effective when given before mating, had less effect in preventing pregnancy in another trial by the same authors when given a few days after coitus

The author has previously (1965) described the effect of subcutaneous administration of 2 mg F6060 or F6066 per male and/or female rat for 6–9 weeks before and 0–3 weeks after contact between males and females As is shown in figs 7 and 8 of the paper cited, both sexes became fertile 3–8 weeks after withdrawal of the substances, the females more rapidly than the males Two mg per animal is approximately equivalent to the highest dose in the experiments described here

During treatment with 2 5 or 12 5 mg/kg F6066, none of the females became pregnant, but 12 out of 20 became pregnant after withdrawal

The two sets of results are thus similar, although sterility appears to continue for a greater time after prolonged treatment than in the present experiments Only one animal treated with F6103 became pregnant after discontinuation It is doubtful if this difference between F6103 and F6066 is other than incidental

With all three substances used, there was a reduction in the number of young from pregnancies begun during treatment After 12.5 mg/kg F6066, a tendency was seen towards reduced litter size in pregnancies begun after discontinuation of the substance

Interrupted pregnancy, with dead foetuses or traces of a decidual reaction, could be established in 4 cases This indicates that an already established pregnancy can be interrupted with at least F6066 and DES

The average number of days from housing of the males with females until the commencement of a successful pregnancy is the same for treated animals as for control animals Thus if a treated animal becomes pregnant during the treat-

Table 4

The antifertility effect in rats of F6066, F6103 and Diethylstilboestrol propionate after daily subcutaneous administration to females (Each group contained 10 animals)

	Number of normally progressing pregnancies begun in each week						Number of animals which were not pregnant at end of experiment	Number of animals with traces of resorbed foetuses at end of experiment	Mean number of young per litter	Estimated number of days *from* mating to pairing
	1	2	3	4	5	6				
Injections	+	+	+	+	−	−				
Male in cage	−	+	+	+	+	+				
F6066 0 5 mg/kg	−	3	1	2	−	1	1	2	9[1]	13
2 5 mg/kg	−	−	−	−	2	5	2	1	*11*	*30*
12 5 mg/kg	−	−	−	−	−	5	5	−	7	33
Oil	−	4	3	3	−	−	−	−	10	10
F6103 0 05 mg/kg	−	4	2	1	1	−	2	−	3[2]	*11*
0 25 mg/kg	−	−	−	−	−	−	10	−	−	−
1 25 mg/kg	−	−	−	−	−	−	10	−	−	−
Oil	−	6	1	3	−	−	−	−	11	9
DES 0 5 µg/kg	−	8	2	−	−	−	−	−	10	5
2 5 µg/kg	−	2	1	−	−	−	6	1	1	6
12 5 µg/kg	−	−	−	−	−	−	10	−		
Oil	−	7	3	−	−	−	−	−	11	6

[1]) The pregnancy begun in the 6th week produced 11 young Two of the females which became pregnant during treatment had 1 and 2 young respectively

[2]) The pregnancy begun in the 5th week produced 10 young

In those groups given 2 5 mg/kg F6066, 0 25 mg/kg F6103, 12 5 µg/kg DES, or higher doses of these substances, no young were delivered (table 4)

The estimated doses required to halve the number of young born per group (ED50) from pregnancies begun during treatment are, for F6066, F6103 and DES respectively, 0 5 mg/kg, ⩽0 05 mg/kg and 1 µg/kg In the group given doses of 0 05 mg/kg F6103, 16 young were delivered from pregnancies begun during treatment compared with 113 in the control group a reduction to 14%

The sterility was not reversible in 2 weeks in groups treated with 2 5 or 12 5 µg/kg DES and 0 25 or 1 25 mg/kg F6103 One animal treated with 0 05 mg/kg F6103 began a normal pregnancy during the first substance-free week After

The effects on fertility and vaginal cytology of twice weekly administration of F6103 or DES to female rats

Introduction

In this series, the number of injections per week was reduced from 7 to 2, given on two consecutive, fixed weekdays, and in addition, vaginal smears were taken daily from each animal. The first dose of the substance was given 3 days after housing of the males with females, when at least some pregnancies should have been initiated. Deviations from the animals' normal sexual cycles could be determined from the vaginal smears, which also enabled establishment of the frequency of mating and possible relations between vaginal cytology, coitus and treatment.

Method

F6103, in doses of 0.1, 0.5 and 2.5 mg/kg, or olive oil alone, 1 ml/kg, was injected subcutaneously twice weekly (two consecutive days and five days without injection) in three month old female rats, a total of 6 injections were given. Each group consisted of 10 females, two females sharing each cage. A male rat was placed in each cage and remained there for three weeks. The course of injections was begun three days after addition of the male and concluded three days after the male was removed. The females were kept under observation for eventual signs of pregnancy for a further three weeks. Pregnant animals (recognized by an increase in abdominal size) were placed in separate cages and the numbers of dead and living young reckoned at birth. The probable date of mating was calculated as the date of birth minus 21 days. Animals which had not given birth to young were sacrificed and their uteri examined.

Vaginal smears were taken during the entire period of treatment and during the first and last weeks of the observation period.

An equivalent series of experiments was carried out with DES, 1, 5 and 25 µg/kg.

Results

The number of young delivered decreased with increasing doses of F6103 and DES. After 0.5 mg/kg and 25 µg/kg, respectively, no young were delivered *at all*. *It* was estimated that reduction to half would be obtained with ≤ 0.1 mg/kg and 8 µg/kg, respectively (tables 5, 6).

ment, this occurs without retardation This suggests that the antifertility effect is not based upon ovulation inhibition

In a simplified form, the present test can, in the same way as the "abortion experiments" on mice and rats described later, be used as a *screening test for* the selection of active substances in a series of chemical syntheses In the form described here, the test makes small demands on personnel and equipment but, due to the length of treatment, the amount of the substance required may be greater than the yield from the first chemical synthesis The amount of a substance required can be reduced to an acceptable size by using mice or by reducing the number of animals per dosage or the number of dosages, and possibly by shortening the period of injection Thus, for example, if two groups of five female rats are given 1 and 5 mg/kg/day, respectively, for one week before housing with males and for two weeks after, 125 mg of an unknown substance will be enough to establish eventual effects The final requirement for a screening method, speed, cannot be fulfilled, as with many other endocrinological methods

The control animals showed on average only one typical oestrous smear in the first week Eight of the 20 control animals became pregnant without oestrus being demonstrated, four of these on the first day of the experiment Since 19 of 20 animals became pregnant in the first week, there was only one oestrous smear in the second week and none thereafter

In the treated groups, 10 to 20 typical oestrous smears were demonstrated in the 70 smears of the first week, but the number of such smears fell to between 0 and 6 per week in the second and third weeks The only exception was the group treated with 25 µg DES, where the frequencies in the second and third weeks were 10/70 and 20/70 In the groups treated with 0 1 mg/kg F6103 or 1 or 5 µg/kg DES, the decrease was partly, but not entirely, due to the number of animals beginning a normally developing pregnancy

When the males were removed at the end of the third week, the frequency of oestrus increased in animals treated with 25 µg/kg DES from 20/70 in the third week to 39/70 in the fourth week and 54/70 in the sixth week This increase was even more strongly expressed in animals treated with 0 5 or 2 5 mg/kg F6103 5/70 to 23/70 to 42/70 and 6/70 to 46/70 to 42/70, respectively A clear increase could be observed during the days immediately following removal of the male, before the last two injections had been given

All 20 control animals became pregnant and delivered young, two of them without sperm being observed in the smears Sperm were not demonstrated more than once in any of the control animals After antifertility doses of F6103 and DES, sperm were demonstrated in normal or increased frequency during all three weeks The matings were not correlated with the days of injection, but a tendency to an increased frequency on the days after drug administration was observed Vaginal bleeding of 1-3 days duration was demonstrated in animals from all groups on the twelfth to sixteenth days of pregnancy In a few treated animals, but in no control animals, the bleeding was followed by a period with a high frequency of oestrus

The average number of days from the start of the experiment to the birth of living young was 26 or 27 days in all groups where young were delivered.

Comments

Hanngren et al (1965b) have briefly described a similar experiment with F6066 but did not include vaginal smears 2 mg/kg F6066 per injection halved the number of young and after 25 mg/kg no young were delivered (table 7)

In 45 day experiments with ethynyl oestradiol, described on page 31, daily vaginal smears were taken to demonstrate the occurrence of sperms in the vagina and the effect of ethynyl oestradiol on the oestrous cycle. No constant pattern was observed in the treated animals, some cycles were a few days longer or shorter than normal for the rat colony Although a high dose of oestrogen was administered, only one animal showed an oestrus in excess of

Table 5

Antifertility effect of F6103 in female rats after subcutaneous injection twice weekly

	F6103 mg/kg			Olive oil 1ml/kg	Injection	Male in cage
	0 1	0 5	2 5			
Number oestrous smears 1st week[1]	17/70	19/70	20/70	9/70	+	+
2nd week	1/70	0/70	3/70	0/70	+	+
3rd week	2/64	5/70	6/70	0/58	+	+
4th week	4/28	23/70	46/70	0/10	–	–
6th week	1/26	42/70	42/70	0/0	–	–
Number of times sperm demon w 1	9	7	14	9		
2	0	4	2	1		
3	1	6	5	0		
Time housing – birth	27 d	–	–	27 d		
Number of animals giving birth	6[2]	0	0	10		
Number of young per litter	7	–	–	11		
Number of animals where no pregnancy demonstrated	2	10	10	0		

[1]) Determined by daily examination of vaginal smears from 10 animals per group, a total of 70 smears per week When pregnancy had been established, smears were discontinued

[2]) In addition one animal with traces of 5 resorbed foetuses at the conclusion of the experiment and another with 3 full term, living foetuses and 4 small decidual knots

Table 6

Antifertility effect of diethylstilboestrol dipropionate in female rats after subcutaneous injection twice weekly (Each group consisted of 10 animals)

	DES μg/kg			Olive oil 1ml/kg	Injection	Male in cage
	1	5	25			
Number oestrous smears 1st week	11/70	10/70	19/70	11/70	+	+
2nd week	1/70	1/70	10/70	1/70	+	+
3rd week	0/55	2/61	20/70	0/57	+	+
4th week	0/20	2/23	39/70	0/4		–
6th week	0/7	10/14	54/70	0/0	–	
Number of times sperm demon w 1	6	8	12	8		
2	1	1	7	0		
3	1	0	5	0		
Time housing – birth	27 d	26 d	–	26 d		
Number of animals giving birth	9	8	0	10		
Number of young per litter	11	10	–	11		
Number of animals where no pregnancy demonstrated	1	2	10	0		

The control animals showed on average only one typical oestrous smear in the first week Eight of the 20 control animals became pregnant without oestrus being demonstrated, four of these on the first day of the experiment. Since 19 of 20 animals became pregnant in the first week, there was only one oestrous smear in the second week and none thereafter

In the treated groups, 10 to 20 typical oestrous smears were demonstrated in the 70 smears of the first week, but the number of such smears fell to between 0 and 6 per week in the second and third weeks The only exception was the group treated with 25 µg DES, where the frequencies in the second and third weeks were 10/70 and 20/70 In the groups treated with 0 1 mg/kg F6103 or 1 or 5 µg/kg DES, the decrease was partly, but not entirely, due to the number of animals beginning a normally developing pregnancy

When the males were removed at the end of the third week, the frequency of oestrus increased in animals treated with 25 µg/kg DES from 20/70 in the third week to 39/70 in the fourth week and 54/70 in the sixth week This increase was even more strongly expressed in animals treated with 0 5 or 2 5 mg/kg F6103 5/70 to 23/70 to 42/70 and 6/70 to 46/70 to 42/70, respectively A clear increase could be observed during the days immediately following removal of the male, before the last two injections had been given

All 20 control animals became pregnant and delivered young, two of them without sperm being observed in the smears Sperm were not demonstrated more than once in any of the control animals After antifertility doses of F6103 and DES, sperm were demonstrated in normal or increased frequency during all three weeks The matings were not correlated with the days of injection, but a tendency to an increased frequency on the days after drug administration was observed Vaginal bleeding of 1–3 days duration was demonstrated in animals from all groups on the twelfth to sixteenth days of pregnancy In a few treated animals but in no control animals, the bleeding was followed by a period with a high frequency of oestrus

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Table 5

Antifertility effect of F6103 in female rats after subcutaneous injection twice weekly

	F6103 mg/kg			Olive oil 1ml/kg	In jec-tion	Male in cage
	0 1	0 5	2 5			
Number oestrous smears 1st week[1])	17/70	19/70	20/70	9/70	+	+
2nd week	1/70	0/70	3/70	0/70	+	+
3rd week	2/64	5/70	6/70	0/58	+	+
4th week	4/28	23/70	46/70	0/10	–	–
6th week	1/26	42/70	42/70	0/0	–	–
Number of times sperm demon w 1	9	7	14	9		
2	0	4	2	1		
3	1	6	5	0		
Time housing – birth	27 d	–	–	27 d		
Number of animals giving birth	6[2])	0	0	10		
Number of young per litter	7	–	–	11		
Number of animals where no pregnancy demonstrated	2	10	10	0		

[1]) Determined by daily examination of vaginal smears from 10 animals per group, a total of 70 smears per week When pregnancy had been established, smears were discontinued

[2]) In addition one animal with traces of 5 resorbed foetuses at the conclusion of the experiment and another with 3 full term, living foetuses and 4 small decidual knots

Table 6

Antifertility effect of diethylstilboestrol dipropionate in female rats after subcutaneous injection twice weekly (Each group consisted of 10 animals)

	DES µg/kg			Olive oil 1ml/kg	In jec tion	Male in cage
	1	5	25			
Number oestrous smears 1st week	11/70	10/70	19/70	11/70	+	+
2nd week	1/70	1/70	10/70	1/70	+	+
3rd week	0/55	2/61	20/70	0/57	+	+
4th week	0/20	2/23	39/70	0/4		–
6th week	0/7	10/14	54/70	0/0		
Number of times sperm demon w 1	6	8	12	8		
2	1	1	7	0		
3	1	0	5	0		
Time housing – birth	27 d	26 d	–	26 d		
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DES, [illegible]
beginning a normally developing pregnancy

When the males were removed at the end of the third week, the frequency of oestrus increased in animals treated with 25 μg/kg DES from 20/70 in the third week to 39/70 in the fourth week and 54/70 in the sixth week This increase was even more strongly expressed in animals treated with 0 5 or 2.5 mg/kg F6103 5/70 to 23/70 to 42/70 and 6/70 to 46/70 to 42/70, respectively A clear increase could be observed during the days immediately following removal of the male, before the last two injections had been given

All 20 control animals became pregnant and delivered young, two of them without sperm being observed in the smears Sperm were not demonstrated more than once in any of the control animals After antifertility doses of F6103 and DES, sperm were demonstrated in normal or increased frequency during all three weeks The matings were not correlated with the days of injection, but a tendency to an increased frequency on the days after drug administration was observed Vaginal bleeding of 1–3 days duration was demonstrated in animals from all groups on the twelfth to sixteenth days of pregnancy In a few treated animals, but in no control animals, the bleeding was followed by a period with a high frequency of oestrus

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Table 6

Antifertility effect of diethylstilboestrol dipropionate in female rats after subcutaneous injection twice weekly (Each group consisted of 10 animals)

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6th week	0/7	10/14	54/70	0/0		-
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All 20 control animals became pregnant and delivered young, two of them without sperm being observed in the smears. *Sperm were not demonstrated* more than once in any of the control animals After antifertility doses of F6103 and DES, sperm were demonstrated in normal or increased frequency during all three weeks The matings were not correlated with the days of injection, but a tendency to an increased frequency on the days after drug administration was observed Vaginal bleeding of 1–3 days duration was demonstrated in animals from all groups on the twelfth to sixteenth days of pregnancy In a few treated animals, but in no control animals, the bleeding was followed by a period with a high frequency of oestrus

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Table 5

Antifertility effect of F6103 in female rats after subcutaneous injection twice weekly

	F6103 mg/kg			Olive oil 1ml/kg	Injection	Male in cage
	0.1	0.5	2.5			
Number oestrous smears 1st week[1])	17/70	19/70	20/70	9/70	+	+
2nd week	1/70	0/70	3/70	0/70	+	+
3rd week	2/64	5/70	6/70	0/58	+	+
4th week	4/28	23/70	46/70	0/10	–	–
6th week	1/26	42/70	42/70	0/0	–	–
Number of times sperm demon w 1	9	7	14	9		
2	0	4	2	1		
3	1	6	5	0		
Time housing – birth	27 d	–	–	27 d		
Number of animals giving birth	6[2])	0	0	10		
Number of young per litter	7	–	–	11		
Number of animals where no pregnancy demonstrated	2	10	10	0		

[1]) Determined by daily examination of vaginal smears from 10 animals per group a total of 70 smears per week When pregnancy had been established, smears were discontinued

[2]) In addition one animal with traces of 5 resorbed foetuses at the conclusion of the experiment and another with 3 full term, living foetuses and 4 small decidual knots

Table 6

Antifertility effect of diethylstilboestrol dipropionate in female rats after subcutaneous injection twice weekly (Each group consisted of 10 animals)

	DES µg/kg			Olive oil 1ml/kg	Injection	Male in cage
	1	5	25			
Number oestrous smears 1st week	11/70	10/70	19/70	11/70	+	+
2nd week	1/70	1/70	10/70	1/70	+	+
3rd week	0/55	2/61	20/70	0/57	+	+
4th week	0/20	2/23	39/70	0/4		–
6th week	0/7	10/14	54/70	0/0		
Number of times sperm demon w 1	6	8	12	8		
2	1	1	7	0		
3	1	0	5	0		
Time housing – birth	27 d	26 d	–	26 d		
Number of animals giving birth	9	8	0	10		
Number of young per litter	11	10	–	11		
Number of animals where no pregnancy demonstrated	1	2	10	0		

mated before the first injection and they probably became pregnant, since none of the control animals needed to mate more than once in order to become pregnant

ED50 for the vaginotrophic effect in castrated female rats can be estimated to 0 02 mg/animal for F6103 and 0 5 μg/animal for DES (table 1) equivalent to about 0 1 mg/kg and 2 5 μg/kg, respectively The antifertility effect of F6103 in the present test (ED50 $\leq$ 0 1 mg/kg) thus seems relatively stronger than the oestrogenic when compared with DES (ED50 for antifertility effect = 8 μg/kg) (table 21) The same relation for F6066, based on information in the literature (tables 1 and 7), shows a similar tendency as for F6103, since the abortifacient ED50 can be estimated as 2 mg/kg and the oestrogenic ED50 as 4 mg/kg.

Table 7

Antifertility in rats with F6066 (from HANNGREN *et al* 1965b)

	No of pregnancies per 6 animals	No of fully developed foetuses per 6 animals
Olive oil	5	51
1 mg/kg/day F6066	4	41
5 mg/kg/day F6066	1	11
25 mg/kg/day F6066	0	0

five days The experiments also showed that the frequency of coitus increased in individual animals, one rat mated 11 times in 15 days In these animals it was impossible to correlate oestrus and mating, since sperm could be observed in pure dioestrous smears At the conclusion of the experiments, a decrease in the weight of the ovaries and thymus and a slight increase in the weight of the uterus were shown Histological examination of the ovaries showed a reduced number of mature follicles but many corpora lutea (WATNICK, GIBSON, VINEGRA & TOLKSDORF 1964)

The demonstrated dose dependent reduction of fertility, despite increased frequency of mating, is thus parallelled by results obtained with F6103 and ethynyl oestradiol

Even in the present experiments, mating has often been seen to occur when animals are in dioestrus or a "suboestrus" which is not apparent in the vaginal smear This may be seen, for example, in table 5, week 2 for animals given 0 5 mg/kg F6103 During the entire week there is not one oestrous smear, yet 4 animals mated on the first and second days after the week's injections

The F6103 treated animals showed a reduced number of oestrous smears in the second and third weeks This is in contrast to the results with ethynyl oestradiol, where the number did not deviate from that of the control group The increased frequency of dioestrus in the second and third weeks after DES and F6103 may depend upon corpus luteum stimulation by these oestrogens In the fourth and sixth weeks, there was a pronounced increase in the frequency of oestrus in the F6103 treated animals This increase was related to the removal of the male at the end of the third week, and not to the injections given 3 and 4 days later This apparently psychological effect cannot at present be explained It was also seen in the groups given 5 and 25 μg DES, although to a less pronounced extent

The effect of F6103 and DES demonstrated in these experiments is not based upon ovulation inhibition, at least not in the start of the experiment It is apparent from the vaginal smears that about half of the experimental animals

intensively with the nidation of the fertilized ovum, induction of experimental decidualization and the possibility of antagonizing it in rats The work forms the background for testing ergocornine in human beings (see page 18)

SHELESNYAK (1960) suggests that after ovulation the genital tract is under gestagen control from the corpora lutea After about 4 days when the eggs are distributed in the uterus there will be a short release of oestrogens which will activate the blastocysts for implantation into the endometrium which is activated also by the oestrogens The activation of the endometrium is, at least partly, provoked by histamine released by the oestrogens. In the rat the oestrogens come from an only partly depressed oestrous cycle In species where ovulation occurs only after coitus (rabbit), the oestrogen or an "x-substance' originates from the blastocyst or its membranes

The trophoblastic activity during the invasion of the decidua is cancer-cell like, it grows highly infiltratively and is phagocytic (SOLOMON 1966)

Our experiments were carried out first in rats, since this is the species whose reproduction is best known As mentioned on page 20, it is known that conventional oestrogens induce abortion in mice and rats if given during the first few days after mating The days of treatment were varied in the present experiments to include later periods, when conventional oestrogens are said to have a poorer effect

Method

Groups of three female rats, about three months old, were each housed with one male rat Each morning vaginal smears were taken and examined for sperm The demonstration of sperm was taken to imply that the rat had mated and was probably pregnant The day on which sperms were found was taken as day 0

The paired females were distributed day by day into three groups until each group contained five animals The rats in two of these groups were treated on two fixed consecutive days with various doses of the test substances dissolved in oil One control group received olive oil All solutions were administered with a stomach tube except EB, which was given subcutaneously The precise distribution of dosages and the days of treatment in relation to day 0 are shown in tables 8 and 9 The animals were most often treated on days 3 and 4 or 11 and 12

The animals were sacrificed 6–8 days after the first day of treatment and their uteri examined The day of sacrifice was not an essential factor since only the number of implantation sites and their appearance were noted Two types of nodes could be distinguished macroscopically *large*, implying a normally developed foetus, and *small*, implying an implantation site with arrested foetal and decidual development When treatment had been given on days 17 and 18, the mother was sacrificed on day 20 and the number of living and dead foetuses counted

The induction of abortion in rats on various days of pregnancy (abortion test)

Introduction

In the two previous sections it was shown that female rats housed with males gave birth to a dose dependently reduced number of young when given F6066, F6103 or DES, daily or twice weekly In the experiments with twice weekly injections, the oestrous cycle and mating were followed with vaginal smears It was established that a number of the animals mated, and therefore were presumably pregnant, before the first injection, yet, nevertheless, those receiving the highest doses gave birth to no young In order to demonstrate which phase or phases in the development of the fertilized egg could be affected by administration of the substances, we decided to use pregnant animals whose date of mating was known

In the rat, the fertilized eggs are transported through the fallopian tubes on days 0–2 (day 0 = day on which sperm demonstrated in smear) They lie in the transition between tubes and uterus or free in the uterus on days 3–4, and implantation occurs on days 5–7 There is developed decidua from day 7 and an organized placenta, communicating with the foetus through an umbilical cord, from day 11 (BROCK & V KREIBING 1964) The tubal transport time is reasonably constant, being 3 to 4 days for all species mentioned here except the dog, which has a tubal transport time of 7 days (SHELESNYAK 1960)

Observations of newly released rat ova suggest that cilial action is of great importance for transport of the egg into the tube, but that the tubal musculature dominates in the control of further transport Cilial motility is stimulated by oestrogens and the contractions of the tubal musculature are also hormone dependent, probably gestagen dependent (AMOROSO & FINN 1962a) ALDEN (1942) has, however, shown that transport can take place independent of ovarian hormones, since he was able to demonstrate apparently normal eggs in the uterus at the correct time in rats ovariectomized after ovulation

Implantation, or nidation, is a two-phased process whereby the growing blastocyst establishes a firm attachment to the uterine wall Firstly, the uterus reacts by the formation of a decidua, and secondly, the decidua is invaded by the activated blastocyst This process requires coordinated action of the uterus and blastocyst with a number of different factors

SHELESNYAK (1960) and his colleagues (SHELESNYAK & KRAICER 1961, BEN-DAVID & SULMAN 1966, LOBEL, LEVY & SHELESNYAK 1966), have been working

ED50 was defined as that dose (calculated on semiloga rithmic paper) which reduced the number of normally developed foetuses to half that in the comparable control group Since the number of pregnant females in the control group could be 4 or 5, a 40–60% reduction was accepted in the estimation of ED50

Results

F6103 exhibited an abortifacient effect when given for a two day period at all stages of pregnancy where it was tested The ED50 for abortion varied between <1 mg/kg for days 1–2 and 16 mg/kg for days 13–14 For a relatively long period, from days 3–4 to days 11–12, ED50 varied little, from 2 till 5 mg/kg (table 8)

ED50 for dienoestrol varied between ≤0 01 mg/kg for days *1–2 and* 0 6 mg/kg for days 13–14 *In the period from days 3–4 to days 11–12, ED50 increased tenfold from* 0 03 mg/kg to 0 3 mg/kg

F6066 was also abortifacient ED50s for days 3–4 and 11–12 were calculated as 20 mg/kg and 50 mg/kg, respectively

In addition to dienoestrol, EB (s c), DES and ethynyl oestradiol were used

Table 9

Number of foetuses and young delivered from rats dosed orally with F6103, dienoestrol or ethynyl oestradiol on days 17 and 18 of pregnancy

Dose per day in mg/kg	Substance	No of young born		No of nodes exceeding no of young born	No of foetuses	
		alive	dead		alive	dead
0 3	Dienoestrol	3	15	15	12	8
1 2		2	12	9	10	3
	Olive oil	0	0	0	51	1
2	Ethynyloestradiol	3	0	7	23	11
8		7	8	11	12	5
	Olive oil	0	0	0	48	0
2	F6103	8	0	5	39	3
8		12	4	7	2	6
	Olive oil	0	0	0	72	0
8	F6103	0	0	0	9	51
32		0	0	0	0	52
	Olive oil	0	0	0	56	5

5 animals per group were used The animals were sacrified day 20 Day of conception = day 0

Table 8

Abortion test in rats The effect of oral administration of F6066, F6103, DES, EB and dienoestrol in oil on various days of pregnancy

Substance	Days treated	ED50	mg/kg	No of normally sized foetuses	% compared with control	No of small nodes	mg/kg	No of normally sized foetuses	% compared with control	No of small nodes	Control: No of normally sized foetuses	Control: No of small nodes
F6103 orally	1–2	<1	4	0	0	0	1	0	0	0	48	0
	3–4	2	8	0	0	0	2	25	47	0	53	0
	5–6	<2	8	0	0	0	2	6	12	8	49	0
	7–8	4	8	7	14	7	2	39	76	13	51	2
	9–10	5	8	14	24	34	2	53	90	13	59	2
	11–12	4	16	13	25	36	4	23	44	29	52	9
	13–14	16	16	32	56	31	4	40	70	21	57	2
	15–16	6	16	8	16	46	4	31	63	10	49	0
Dienoestrol orally	1–2	≤0 01	0 04	0	0	0	0 01	21	36	0	58	0
	3–4	0 03	0 08	0	0	6	0 02	43	75	8	57	0
	5–6	0 03	0 08	0	0	0	0 02	42	76	1	55	0
	7–8	0 04	0 08	7	15	7	0 02	37	79	8	47	0
	9–10	>0 3	0 32	48	75	16	0 08	48	75	14	64	1
	11–12	0 3	0 32	29	55	21	0 08	38	72	22	53	3
	13–14	0 6	0 64	32	60	17	0 16	51	96	11	53	1
F6066 orally	3–4	20	40	0	0	7	10	53	139	0	38	0
	11–12	50	50	36	55	23	–	–	–	–	65	3
EB s c	3–4	0 006	0 016	0	0	0	0 004	43	72	0	60	0
	11–12	0 006	0 01	15	26	44	0 0025	59	102	5	58	4
DES orally	3–4	0 03	0 05	0	0	20	0 02	34	77	0	44	0
	11–12	0 3	0 4	18	30	23	0 1	55	90	6	61	1
Ethynyloestradiol orally	3–4	0 2	0 32	0	0	24	0 08	56	[illegible]	[illegible]	[illegible]	[illegible]
	11–12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Injection of HCG, prolaction, progesterone or pregnenolone concomitantly with administration of F6066 to pregnant rats

Introduction

In an attempt to distinguish between the importance of uterine and ovarian factors in the induction of abortion, the abortion test in rats was modified by concomitant administration of F6066 and hormones of hypophyseal, placental or ovarian origin which might have a pregnancy maintaining effect.

Method

The abortion test in rats was used as a basis for these experiments The substances which might possibly offer protection against the induction of abortion (HCG, prolactin, progesterone, pregnenolone) were injected s c on days 3 and 4, concomitantly with administration of 50 mg/kg F6066 in oil per os The dosage of F6066 chosen was taken to be 50–100% effective as an abortifacient Administration of progesterone with F6066 was also carried out on days 11–12 instead of days 3 4

The HCG and prolactin preparations were dissolved in 0 9% NaCl, the progesterone preparation was diluted with olive oil and pregnenolone was dissolved in olive oil As mentioned on page 26, commercially available HCG and prolactin preparations were used

The precise distribution of dosages and days of injection is shown in table 10

Results

The administration of progesterone, pregnenolone or preparations containing HCG or prolactin did not extinguish the abortifacient effect of 50 mg/kg F6066 given (table 10)

The doses of HCG used seemed themselves to have a detrimental effect upon the sensitive hormone balance prevailing on days 3–4, whilst the number of foetuses was affected to only a minor degree by progesterone, pregnenolone and prolactin

as reference substances, and all three showed an abortifacient effect when given on days 3–4 or 11–12 ED50s for EB were 0 006 mg/kg at both periods, ED50s for DES were 0 03 and 0 3 mg/kg, respectively, and ED50s for *ethynyl oestradiol* were 0 2 and 0 6 mg/kg, respectively

When 32 mg/kg F6103 was given on days 17–18 of pregnancy, not a single foetus remained alive on day 20 (table 9) An effect on the number of surviving foetuses was also observed with the two other dosages tested After administration of 2 mg/kg, the lowest of these, a number of young were born Some of these young were dead and others were presumably eaten up before the mother was sacrificed, since there were a number of implantation sites in the uterus which could not be accounted for by living or dead young Administration of 8 mg/kg led to the death of nearly all the foetuses in one experiment, whilst in another the results were similar to those obtained with 2 mg/kg Treatment with 0 3 or 1 2 mg/kg dienoestrol and 2 or 8 mg/kg ethynyl oestradiol produced, in principle, the same changes as 2 mg/kg F6103 After administration of 32 mg/kg F6103 the foetuses in the uterus were white, the uterus was wrapped tightly around them, and amniotic fluid was lacking In the animals treated with 2 mg/kg F6103, and those given dienoestrol or ethynyl oestradiol, the foetuses were pale but amniotic fluid was present

Comments

The experiments described here have been termed "abortion tests" as in the similar experiments with other species The term "abortion" may be defined as interruption of pregnancy at such an early stage that the foetus has no chance of survival outside the uterus Abortion is followed either by expulsion of the foetus and placenta (abortus completus) or retention of the dead foetus in the uterus (abortus retentus, missed abortion) As can be seen from table 8, both types of abortion occur in the present material Abortus completus was most often found after administration of the substances at an early stage of pregnancy, whilst an increased frequency of abortus retentus (small nodes) was found after treatment from days 7–8 onwards with F6103 or dienoestrol

ED50 for abortion with various substances tested on various days is shown in table 21, where these doses are compared with the oestrogenicity of the substances when given subcutaneously In addition, ratios are calculated between the ED50s for abortion on days 3–4 and 11–12, respectively, and between each of these ED50s and oestrogenicity

Repeating the administration of F6103 days 13–14 ED50 was found to 8 mg/kg

Injection of HCG, prolaction, progesterone or pregnenolone concomitantly with administration of F6066 to pregnant rats

Introduction

In an attempt to distinguish between the importance of uterine and ovarian factors in the induction of abortion, the abortion test in rats was modified by concomitant administration of F6066 and hormones of hypophyseal, placental or ovarian origin which might have a pregnancy maintaining effect.

Method

The abortion test in rats was used as a basis for these experiments The substances which might possibly offer protection against the induction of abortion (HCG, prolactin, progesterone, pregnenolone) were injected s c on days 3 and 4, concomitantly with administration of 50 mg/kg F6066 in oil per os The dosage of F6066 chosen was taken to be 50–100% effective as an abortifacient. Administration of progesterone with F6066 was also carried out on days 11–12 instead of days 3–4

The HCG and prolactin preparations were dissolved in 0 9% NaCl, the progesterone preparation was diluted with olive oil and pregnenolone was dissolved in olive oil As mentioned on page 26, commercially available HCG and prolactin preparations were used

The precise distribution of dosages and days of injection is shown in table 10

Results

The administration of progesterone, pregnenolone or preparations containing HCG or prolactin did not extinguish the abortifacient effect of 50 mg/kg F6066 given (table 10)

The doses of HCG used seemed themselves to have a detrimental effect upon the sensitive hormone balance prevailing on days 3–4, whilst the number of foetuses was affected to only a minor degree by progesterone, pregnenolone and prolactin

Table 10

Abortion test in rats combination of 50 mg/kg F6066 orally and HCG, prolactin, progesterone or pregnenolone subcutaneously

	Days treated	Units/kg or mg/kg s c	mg/kg F6066 *orally*	No of normally sized foetuses	% compared with control	No of small nodes
Progesterone	3–4	125	50	0	0	23
	,,	Olive oil	0	61		0
	3–4	25	50	16	26	10
	,,	5	50	18	30	5
	,,	Olive oil	0	61		0
	3–4	25	0	63	97	0
	,,	Olive oil	0	65		0
	11–12	0	50	36	55	23
	,,	125	50	27	42	25
	,,	Olive oil	0	65		3
Pregnenolone	3–4	25	50	0	0	11
	,,	5	50	0	0	12
	,,	Olive oil	0	60		0
	3–4	25	0	56	86	0
	,,	Olive oil	0	65		0
	3–4	125	50	0	0	13
	,,	Olive oil	0	61		0
Prolactin	3–4	125	0	50	89	0
	,,	125	50	0	0	15
	,,	Olive oil	0	56		0
	3–4	25	0	53	113	0
	,,	25	50	0	0	15
	,,	Olive oil	0	47		0
HCG	3–4	125	50	0	0	9
	,,	25	50	0	0	0
	,,	Olive oil	0	61		0
	3–4	125	0	36	63	0
	,,	0	50	23	40	10
	,,	Olive oil	0	57		0

Progesterone and pregnenolone were given in mg/kg dissolved in oil, HCG and Prolactin in units/kg dissolved in 0 9 % NaCl

Comments

In no case could the hormones given hinder abortion The explanation of this is as yet unknown MARTIN (1963) was unable to extinguish the abortifacient effect of oestradiol with progesterone injection, despite a clear gestagen influence upon the vaginal cytology

The induction of abortion in mice on various days of pregnancy

Introduction

Since studies had been made of ^{14}C-F6066's distribution in the mouse (page 74), it was felt desirable to carry out an abortion test in mice, designed in the same way as in the rat experiments

The physiology of early pregnancy in the mouse is regarded to be very similar to that in the rat, although implantation takes place 1–2 days earlier The significance of histamine for the initiation of the decidual reaction appears to be unknown for the mouse, in contrast to the rat

Method

Groups of 2 3 female mice were each housed with one male The vagina was inspected every morning and the presence of a vaginal plug taken as an indication that mating had occurred This day was taken as day 0 The mated animals were distributed daily into three groups until each group contained 5 animals The mice in two groups were given the test substance, dissolved in oil, by stomach tube Those in the third group received 10 ml/kg olive oil by the same route

The mice were treated on days 3–4 or 11–12 and sacrificed 6–8 days later The uteri were inspected, the number of large and small nodes was counted, and ED50 was calculated in the way described for the rat abortion test

The doses used of dienoestrol ethynyl oestradiol, DES, EB, F6066 and F6103 are shown in table 11

Results

Both F6066 and F6103 induce abortion *in the mouse 2 5 and 10 mg/kg F6056* given on days 3–4 gave a reduction of 21% and 81%, respectively, in the number of normally developed foetuses as compared with the control group After 0 25 and 1 mg/kg F 6103 on days 3–4, reductions of 42% and 100% were found ED50s for dienoestrol, ethynyloestradiol DES and EB on days 3–4 were 3, 10, 10 and 2 µg/kg, respectively ED50s for DES, EB and F6103 on days 11–12 were 200 µg/kg, 40 µg/kg and 2 mg/kg, respectively, for dienoestrol, ethynyl

Table 11

Abortion test in mice The effect of oral administration of F6066, F6103, DES, EB, dienoestrol and ethynyloestradiol on various days of pregnancy

Substance	Days treated	ED50	mg/kg	No of normally sized foetuses	% compared with control	No of small nodes	mg/kg	No of normally sized foetuses	% compared with control	No of small nodes	Control: No of normally sized foetuses	Control: No of small nodes
F6103	3–4	0 3	1	0	0	0	0 25	22	58	4	38	1
	11–12	2	8	0	0	32	2	22	56	6	39	2
Dienoestrol	3–4	*0 003*	0 004	17	43	0	0 001	28	70	0	40	0
	11–12	>0 2	0 16	38	83	3	0 04	35	76	0	46	1
F6066	3–4	5	10	8	19	5	2 5	34	79	4	43	2
	11–12	>80	80	28	70	2	20	29	73	4	40	4
EB	3–4	0 002	0 004	0	0	14	0 001	30	75	7	40	1
	11–12	0 04	0 16	10	29	4	0 04	18	51	0	35	2
DES	3–4	*0 01*	0 04	0	0	0	0 01	23	56	7	41	0
	11–12	0 2	0 4	6	12	0	0 1	49	98	1	50	0
Ethynyloestradiol	3–4	0 01	0 02	4	7	12	0 005	57	106	2	54	0
	11–12	>1 6	1 6	40	91	4	0 4	42	95	1	44	0

oestradiol and F6066, ED50 was >200 µg/kg, >2 mg/kg and >80 mg/kg (table 11) 80 mg/kg F6066 gave a 30% reduction in the number of normally developed foetuses

Comments

These experiments show that the abortifacient effect demonstrated for F6066 and F6103 in rats can also be obtained in mice The effective ED50 dosages per kg body weight on days 3–4 are 2–10 times smaller for the mouse than for the rat As in the rat, F6103 is relatively more active than conventional oestrogens on days 11–12 An ED50 value for F6066 on days 11–12 was not reached This is probably due to the animals' inability to resorb the large doses given, and should not be taken to imply a difference in the mechanisms of action of F6103 and F6066

The general discussion includes a calculation of ratios between the abortifacient and oestrogenic effects and between the ED50s for days 11–12 and 3–4, respectively (table 21)

The induction of abortion in rabbits on various days of pregnancy

Introduction

LARSSON (personal communication) observed that ca 10% of a given amount of F6103, administered in oil, orally or s c , could be demonstrated in the urine of rats within 96 hours after administration In rabbits, on the other hand, 60% of the F6103 given (orally in oil) could be demonstrated within the same period of time It is possible that the rabbit excretes a smaller proportion of the administered substance in the faeces in the same time period It was one intention of these experiments to investigate whether the dose relation between the rat and the rabbit was of the same order as the difference in urinary excretion

By sacrificing the rabbits one day before expected term and examining the offspring, the foetuses could be used as a species control for the demonstration of eventual teratogenic effects

Method

Mature female rabbits were paired under supervision with males of known fertility The date of mating was taken as day 0 The females were thenceforward housed singly and were given F6103 (0 2–12 8 mg/kg, dissolved in oil) or the control solution daily by stomach tube Three dosage periods were chosen – days 1–9, 10–18 and 19–27 – and two animals were used in each group The rabbits were sacrificed on day 28 and the number of living and dead foetuses and of implantation sites with abnormal development was noted The living foetuses were weighed and their thoracic and abdominal organs were examined under a stereomicroscope The foetal skeleton was stained with alizarin and the bones counted The foetal examinations were carried out in the same way as for rats and mice, and the method is more fully described in the section dealing with teratogenic investigations ED50 for abortion was calculated in the way described for the rat abortion test

Results

F6103 was capable of interrupting pregnancy in all three dosage periods tested (table 12) The reduction in the number of foetuses was dose dependent

Table 12

Antifertility and teratogenic tests with F6103 in rabbits

mg/kg per day	day	No of normally pregnant animals	Living foetuses		Dead foetuses		Average weight of foetuses g	% compared with control[1]
			♀	♂	5–30 mm	> 30 mm		
K 1 3 2	1–9	1	2	–	–	–	51	12
3 2	10–18	2	4	9	1	3	29	76
3 2	19–27	1[2]	4	5	–	1	23	53
Oil	1–9	2	8	9	–	1	37	
12 8	1–9	–	–	–	–	–	–	–
12 8	10–18	–[3]	–	–	–	–	–	–
12 8	19–27	–[4]	–	–	–	–	–	–
Oil	19–27	2	12	5	–	–	36	
K 2 0 2	1–9	2	8	8	1	–	37	94
0 2	10–18	1[5]	6	2	–	–	39	47
0 2	19–27	2	6	5	–	1	30	65
Oil	1–9	2	10	8	2	1	33	
0 8	1–9	1	8	2	–	–	31	59
0 8	10–18	2	6	6	1	3	33	71
0 8	19–27	2	8	11	–	–	27	112
Oil	19 27	2	5	11	–	–	35	

[1]) Calculated from 4 control animals in K 1 and K 2 respectively
[2]) The second animal had 10 partially resorbed foetuses in the uterus.
[3]) Both animals with partially resorbed foetuses in the uterus 8 and 9, respectively
[4]) Both animals with partially resorbed foetuses in the uterus 7 each
[5]) The second animal had 6 small nodes in the uterus
Mother sacrificed on day 28

ED50s for abortion were 1, 5 and 3 mg/kg for periods 1–9, 10–18 and 19–27 days, respectively

A tendency towards an increase in the number of dead foetuses in the uterus was observed after F6103 treatment The eight rabbits treated with olive oil had a total of 4 dead foetuses, or 0 5 per mother In the 12 rabbits treated with F6103 on days 10–18 or 19–27 which contained foetuses at all, nine dead foetuses were found, or 0 8 per mother A reduction in the weight of the living foetuses was observed after treatment on days 19–27, foetuses from mothers treated with 3 2 mg/kg weighed an average of 23 grams compared with an average of 37 grams for the controls This difference was statistically significant ($p < 0\ 001$; Student's *t*-test)

The induction of abortion in rabbits on various days of pregnancy

Introduction

LARSSON (personal communication) observed that ca 10% of a given amount of F6103, administered in oil, orally or s c, could be demonstrated in the urine of rats within 96 hours after administration In rabbits, on the other hand, 60% of the F6103 given (orally in oil) could be demonstrated within the same period of time It is possible that the rabbit excretes a smaller proportion of the administered substance in the faeces in the same time period It was one intention of these experiments to investigate whether the dose relation between the rat and the rabbit was of the same order as the difference in urinary excretion

By sacrificing the rabbits one day before expected term and examining the offspring, the foetuses could be used as a species control for the demonstration of eventual teratogenic effects

Method

Mature female rabbits were paired under supervision with males of known fertility The date of mating was taken as day 0 The females were thence forward housed singly and were given F6103 (0 2–12 8 mg/kg, dissolved in oil) or the control solution daily by stomach tube Three dosage periods were chosen – days 1–9, 10–18 and 19–27 – and two animals were used in each group The rabbits were sacrificed on day 28 and the number of living and dead foetuses and of implantation sites with abnormal development was noted The living foetuses were weighed and their thoracic and abdominal organs were examined under a stereomicroscope The foetal skeleton was stained with alizarin and the bones counted The foetal examinations were carried out in the same way as for rats and mice, and the method is more fully described in the section dealing with teratogenic investigations ED50 for abortion was calculated in the way described for the rat abortion test

Results

F6103 was capable of interrupting pregnancy in all three dosage periods tested (table 12) The reduction in the number of foetuses w

Table 12

Antifertility and teratogenic tests with F6103 in rabbits

mg/kg per day	day	No of normally pregnant animals	Living foetuses		Dead foetuses		Average weight of foetuses g	% compared with control[1]
			♀	♂	5–30 mm	> 30 mm		
K 1 3 2	1–9	1	2	–	–	–	51	12
3 2	10–18	2	4	9	1	3	29	76
3 2	19–27	1[2]	4	5	–	1	23	53
Oil	1–9	2	8	9	–	1	37	
12 8	1–9	–	–	–	–	–	–	–
12 8	10–18	–[3]	–	–	–	–	–	–
12 8	19–27	–[4]	–	–	–	–	–	–
Oil	19–27	2	12	5	–	–	36	
K 2 0 2	1–9	2	8	8	1	–	37	94
0 2	10–18	1[5]	6	2	–	–	39	47
0 2	19–27	2	6	5	–	1	30	65
Oil	1–9	2	10	8	2	1	33	
0 8	1–9	1	8	2	–	–	31	59
0 8	10–18	2	6	6	1	3	33	71
0 8	19 27	2	8	11	–		27	112
Oil	19–27	2	5	11	–	–	35	

[1]) Calculated from 4 control animals in K 1 and K 2 respectively
[2]) The second animal had 10 partially resorbed foetuses in the uterus
[3]) Both animals with partially resorbed foetuses in the uterus 8 and 9, respectively
[4]) Both animals with partially resorbed foetuses in the uterus 7 each
[5]) The second animal had 6 small nodes in the uterus
Mother sacrificed on day 28

ED50s for abortion were 1, 5 and 3 mg/kg for periods 1–9, 10–18 and 19–27 days, respectively

A tendency towards an increase in the number of dead foetuses in the uterus was observed after F6103 treatment The eight rabbits treated with olive oil had a total of 4 dead foetuses, or 0 5 per mother In the 12 rabbits treated with F6103 on days 10–18 or 19–27 which contained foetuses at all, nine dead foetuses were found or 0 8 per mother A reduction in the weight of the living foetuses was observed after treatment on days 19–27, foetuses from mothers treated with 3 2 mg/kg weighed an average of 23 grams compared with an average of 37 grams for the controls This difference was statistically significant ($p < 0\ 001$, Student's t-test)

One of the control foetuses exhibited serious malformations in the head and neck One of the foetuses from an animal treated with F6103 showed divided *sternal bones on study of the alizarin stained skeleton*

Comments

The demonstrated ED50s for abortion in the rabbit during the three periods lie between 1 and 5 mg/kg given daily for 9 days

As shown in tables 17 and 18, points R 11 and M 10, ED50 for the induction of abortion *with F6103 in rats on days 10–19 is 0 1 mg/kg* and mice treated on days 9–17 with 0 1 mg/kg F6103 contained 27% fewer foetuses than the control animals Even if treatment with F6103 during the last nine days of pregnancy in rats covers a proportionately longer period than nine days in rabbits, this difference is not sufficiently great to account to more than a minor extent for a tenfold difference in ED50 for abortion A difference in the rate of metabolism (LARSSON 1965), better explains the difference in dosage level, since the rabbit metabolizes and excretes F6103 about 6 times faster than the rat

ED50 for abortion during days 1–9 is lower than in the other two periods This may be due to an acceleration of tubal transport during the first few days, dependent upon F6103's oestrogenicity It is not at present possible to say *whether the lower ED50 for days 19–27 (3 mg/kg)* as compared with days 10–19 (5 mg/kg) is an experimental artefact dependent upon the small number of animals used per dose, or whether there is another factor involved

The induction of abortion in dogs on various days of pregnancy

Introduction

For several reasons, interruption of pregnancy in bitches is often desired by their owners, most often because the mate is unknown or of another strain The experiments described in this section were performed to decide whether F6103 could be of interest within veterinary practice for this indication In addition to this practical aspect, the experiments may be regarded as a species control of F6103's effects, possible side effects and eventual teratogenic effects It may, however, be questioned whether experiments in reproductive physiology performed in dogs are as significant from the human medical point of view as experiments performed in rats and mice, since the dog has a placenta endotheliochorialis whilst both rats and men have a placenta haemochorialis (NIELSEN 1965) Furthermore, the physiology of early pregnancy seems to have been far less examined in the dog than in the rat

Method

Beagle bitches weighing about 10 kg were used from the same stock as used in the previously published experiments on induction of ovulation with F6066 (EINER JENSEN 1967b) Two of the animals used here (nos 16 & 19) received F6066 in the experiments on ovulation induction and two more (11 & 13) formed part of the control group in that series Bitches 11, 12 and 16 were used twice in the abortion experiments, receiving first 100 and then 300 mg/day

Some of the bitches had earlier borne a litter, whilst others had not, as show in table 13 Mating with a fertile male was arranged on the 11th to 14th day after the first day of heat and repeated two days later The first mating was taken as day 0 of pregnancy and the animals received F6103 on three fixed days reckoned from this day The substance was given orally in gelatin capsules or subcutaneously dissolved in oil to a concentration of 10 mg/ml Capsules were given on days 2, 3 & 4 in doses of 100 or 300 mg per animal per day, and on days 6, 7 and 8 in a dose of 100 mg per animal per day The daily dose was divided between one morning and one evening administration On days 10, 11 and 12, F6103 was given as a single, daily, subcutaneous injection of 5 or 10 mg/kg dissolved in oil The animals were clinically examined both before and

One of the control foetuses exhibited serious malformations in the head and neck. One of the foetuses from an animal treated with F6103 showed divided sternal bones on study of the alizarin stained skeleton.

Comments

The demonstrated ED50s for abortion in the rabbit during the three periods lie between 1 and 5 mg/kg given daily for 9 days.

As shown in tables 17 and 18, points R 11 and M 10, ED50 for the induction of abortion with F6103 in rats on days 10–19 is 0.1 mg/kg and mice treated on days 9–17 with 0.1 mg/kg F6103 contained 27% fewer foetuses than the control animals. Even if treatment with F6103 during the last nine days of pregnancy in rats covers a proportionately longer period than nine days in rabbits, this difference is not sufficiently great to account to more than a minor extent for a tenfold difference in ED50 for abortion. A difference in the rate of metabolism (LARSSON 1965), better explains the difference in dosage level, since the rabbit metabolizes and excretes F6103 about 6 times faster than the rat.

ED50 for abortion during days 1–9 is lower than in the other two periods. This may be due to an acceleration of tubal transport during the first few days, dependent upon F6103's oestrogenicity. It is not at present possible to say whether the lower ED50 for days 19–27 (3 mg/kg) as compared with days 10–19 (5 mg/kg) is an experimental artefact dependent upon the small number of animals used per dose, or whether there is another factor involved.

after administration of the substance, but no laboratory investigations were carried out. Immediately after a completed pregnancy and delivery, the pups were weighed and examined for possible signs of malformation

Results

2 × 50 mg/animal/day F6103, given orally in gelatin capsules for three days on days 6, 7 and 8 of pregnancy, could not hinder the completion of a normal pregnancy (table 13) One of the two animals given 100 mg F6103 on days 2, 3 and 4 (no 35) did not complete a normal pregnancy but gave a red discharge from the vagina three weeks after treatment This animal was treated with ergot alkaloid (Secatotal ®), the discharge ceased after one week, and the animal came into heat six months later After the oral dose was increased to 300 mg F6103 per day, none of the animals treated on days 2, 3 and 4 became pregnant Some swelling and reddening of the vagina could be observed Bitch 13 showed an increase in abdominal size in the weeks before expected term, but no pups were born After subcutaneous injection of 5 or 10 mg/kg F6103 on days 10, 11 and 12 or 10 mg/kg F6103 on days 15, 16 and 17, no young were born In both animals, swelling and reddening of the vagina were observed In addition, a little yellow white pus was observed in the vagina of the animal which received 10 mg/kg on days 10, 11 and 12 during the first week after treatment No malformed pups were observed

Comments

These experiments may be regarded only as an indication that F6103 is an abortifacient in dogs The results suggest that if sufficiently large doses of F6103 are used, the pregnancy is interrupted even if treatment is given as late as 10, 11 and 12 (15, 16 and 17) days after mating The oestrogenic effect of the substance is evident in bitches, since the perineum becomes swollen and the vaginal epithelium becomes hyperaemic It is not known if bitch 35, which gave a red discharge from the vagina three weeks after treatment, was pregnant, but this seems probable If this is the case, the bleeding was probably an indication of a delayed and not entirely uncomplicated abortion Bitch 13, which in one case showed an increase in abdominal size before expected term, had presumably had her pregnancy interrupted at the time of treatment but then gone into a state of pseudopregnancy

Oestradiol and stilboestrol are used in veterinary practice to induce abortion in the bitch They have without doubt an effect but it has not been possible to find a published controlled trial Because of this and the limited material obtained with F6103 it is not possible to decide whether F6103's action in the bitch is based only upon its oestrogenic properties or upon something else

Table 13

Induction of abortion in bitches by administration of F6103 on various days of pregnancy
(Body weight 9–13 kg)

Bitch no	Dose in mg per animal per day	orally s c	No earlier whelpings	age in months	Time in days			No pups		Average weight g	
					1st day of heat – 1st mating	1st mating – 1st treatment	1st mating – whelping	♂	♀		
11	100	orally	1	36	12	6–7–8	62	4	3	300	{ 5 died after 1–2 weeks
13	100	orally	3	35	13	6–7–8	63	2	4	320	
16	100	orally	1	36	12	6–7–8	63	4	2	350	{ 2 died after 1–2 weeks
19	100	orally	1	22	14	2–3–4	66	1	4	290	
35	100	orally	0	13	13	2–3–4	–	0	0		{ red discharge from vagina 3 weeks after mating
13	300	orally	4	42	11	2–3–4	–	0	0		
11	300	orally	2	41	12	2–3–4	–	0	0		
16	300	orally	2	43	13	2–3–4	–	0	0		
32	10 mg/kg	s c	2	26	12	10–11–12	–	0	0		
21	5 mg/kg	s c	0	27	12	10–11–12	–	0	0		
13	10 mg/kg	s c	4	48	11	15–16–17	–	0	0		

after administration of the substance, but no laboratory investigations were carried out Immediately after a completed pregnancy and delivery, the pups were weighed and examined for possible signs of malformation

Results

2 × 50 mg/animal/day F6103, given orally in gelatin capsules for three days on days 6, 7 and 8 of pregnancy could not hinder the completion of a normal pregnancy (table 13) One of the two animals given 100 mg F6103 on days 2, 3

alkaloid (Secatotal ®), the discharge ceased after one week, [illegible] came into heat six months later After the oral dose was increased to 300 mg F6103 per day none of the animals treated on days 2, 3 and 4 became pregnant. Some swelling and reddening of the vagina could be observed Bitch 13 showed an increase in abdominal size in the weeks before expected term, but no pups were born After subcutaneous injection of 5 or 10 mg/kg F6103 on days 10, 11 and 12 or 10 mg/kg F6103 on days 15, 16 and 17, no young were born In both animals swelling and reddening of the vagina were observed In addition, a little yellow white pus was observed in the vagina of the animal which received 10 mg/kg on days 10 11 and 12 during the first week after treatment No malformed pups were observed

Comments

These experiments may be regarded only as an indication that F6103 is an abortifacient in dogs The results suggest that if sufficiently large doses of F6103 are used the pregnancy is interrupted even if treatment is given as late as 10, 11 and 12 (15 16 and 17) days after mating The oestrogenic effect of the substance is evident in bitches since the perineum becomes swollen and the vaginal epithelium becomes hyperaemic It is not known if bitch 35, which gave a red discharge from the vagina three weeks after treatment was pregnant, but this seems probable If this is the case the bleeding was probably an indication of a delayed and not entirely uncomplicated abortion Bitch 13, which in one case showed an increase in abdominal size before expected term, had presumably had her pregnancy interrupted at the time of treatment but then gone into a state of pseudopregnancy

Oestradiol and stilboestrol are used in veterinary practice to induce abortion in the bitch They have without doubt an effect but it has not been possible to find a published controlled trial Because of this and the limited material obtained with F6103 it is not possible to decide whether F6103 s action in the bitch is based only upon its oestrogenic properties or upon something else

It is, however, striking that we have not observed a proper heat in the days after F6103 treatment

Calculated in mg/kg, the doses of F6103 used here (10–30 mg/kg) are 3–8 times higher than the ED50 for rats on days 11–12 Since no investigations have been made in the dog on the resorption and metabolism of F6103, it is not possible to say whether this depends upon a difference in the rate of metabolism, as was the case for the rabbit

Teratological studies of F6066 and F6103 in mice and rats

Introduction

Teratological investigations of abortive substances form an important part of the animal experimental studies which should be undertaken before the eventual use of such substances by human beings

Only during the last 25 years, beginning with GREGG's (1941) observations on the effect of maternal rubella infection upon the embryo, and culminating with the Thalidomide disaster, has it been realized that environmental factors may be important in relation to human congenital defects Earlier, however, it was known that malformations could be produced experimentally in animals by, for example, roentgen irradiation or vitamin A deficiency

Efforts to correlate experimental findings with clinical experience have increased in recent years, and methodological studies have been carried out on a large scale

100% security for humans cannot be expected from animal experiments, but it may be hoped that a substance which shows no evidence of teratogenic effects in experiments on several animal species offers a lower risk than a substance which is a potent teratogen in two or more animal species

Whilst this line of argument is presumably acceptable to most people, the problem nevertheless becomes complicated when signs of teratogenic properties of substances are found in animal experiments If it is argued that all such substances should be rejected, then it must be borne in mind that acetylsalicic acid, the barbiturates, phenacetin, meclizine, imipramine, tetracycline, cortisone and several other widely used drugs are teratogenic in animal experiments (KALLÉN 1967)

Method

All the female animals were virgin but males known to be fertile were used During the pairing period the animals were kept in plastic cages, 1 male and 2-4 females in each cage Every morning at 8 a m vaginal smears were taken from the female rats for microscopic examination for spermatozoa In mice the presence of a plug in the vagina was taken as evidence that coitus had occurred

It is, however, striking that we have not observed a proper heat in the days after F6103 treatment.

Calculated in mg/kg, the doses of F6103 used here (10-30 mg/kg) are 3-8 times higher than the ED50 for rats on days 11-12. Since no investigations have been made in the dog on the resorption and metabolism of F6103, it is not possible to say whether this depends upon a difference in the rate of metabolism, as was the case for the rabbit.

Table 15

Antifertility and teratogenic tests with F6066 in mice

Test	Substance adm by stom tube day	Dose/day mg/kg	Substance	No of pregn / no of rats	Young *born* before killed	Total no of liv foet per group		Average weight of liv foet killed on day 20	Total no of dead foet per *group*		Dead foet per pregn	Anogenital distance mm		Foet as %age of contr
						♀	♂		> 3 mm	< 3 mm		♀	♂	
M 1	6–7–8	10 ml/kg	Olive oil	6/10	9	21	32	1 34	–	6	1 0			
	6 7 8	32	F6066	1/10	–	4	5	–	–	–	–			15
	6–7–8	64	*F6066*	2/10	–	7	9	1 25	–	–	–			26
	6–7 8 s c	100	Trypan blue	6/10	*8*	18	27	1 29	1	10	1 8			85
M 2	1–2	10 ml/kg	*Olive oil*	1/4	–	4	2	1 17	–	–	–			
	1–2	64	F6066	1/5	–	1	0	0 75	–	–	–			17
	3–4	64	F6066	0/5	–	–	–	–	–	–	–			–
	5–6	64	F6066	0/5	–	–	–	–	–	–	–			–
	7–8	64	F6066	2/5		7	8	1 22	–	2	1 0			250
M 3	1–2	10 ml/kg	Olive oil	5/6	–	24	22	2 8	1	3	0 8	2 1	3 1	
	1–2	64	F6066	1/6	–	–	–	–	2	3	5 0	–	–	–
	3–4	64	F6066	0/6	–	–	–	–	–	–	–	–	–	–
	5–6	64	F6066	0/6	–	–	–	1 7	–	–	–	–	–	–
	7–8	64	*F6066*	2/6	5	0	2	11	5	–	5 0	–	3 3	15
M 4	6–7–8	10 ml/kg	Olive oil	6/8	–	20	33	1 7	–	8	1 3	2 1	3 0	
	6–7–8	8	F6066	3/9	–	4	4	1 41	4	3	2 3	2 0	3 1	15
	6–7–8	16	F6066	5/8	–	19	20	1 22	2	10	2 4	2 0	3 1	74
	6–7–8	32	F6066	2/9	–	5	4	1 19	2	3	2 5	2 1	3 0	17
M 5	1–2	10 ml/kg	*Olive oil*	5/8	–	18	16	1 34	3	6	1 8	2 2	3 2	
	1–2	16	F6066	1/8	–	2	3	0 63	–	2	2 0	1 8	2 5	15
	3–4	16	F6066	0/8	–	–	–	–	–	–	–	–	–	–
	5–6	16	F6066	2/8	–	5	8	1 23	5	1	3 0	2 3	3 1	38
	7–8	16	*F6066*	5/8	–	16	17	1 23	2	9	2 2	2 0	3 0	97

Table 14

Antifertility and teratogenic tests with F6066 in rats

Test	Substance adm by stom tube day	Dose/day mg/kg	Substance	No of pregn / no of rats	Young born before killed	Total no of liv foet per group		Average weight of liv foet killed on day 20	Total no of dead foet per group		Dead foet per pregn	Anogenital distance mm		Foet as %age of contr
						♀	♂		> 3mm	< 3mm		♀	♂	
R 1	7 8 9	1 ml/kg	Olive oil	6/6		30	28	3 19	1	9	1 6			
		2	F6066	5/6		18	31	3 24	–	5	1 0			84
		4	F6066	4/6		25	16	3 88	1	1	0 5			71
		8	F6066	5/5		23	29	3 24	1	7	1 6			90
R 2	7 8 9	1 ml/kg	Olive oil	9/10		56	43	3 11	1	6	0 8			
		8	F6066	9/10		36	49	3 13	–	12	1 3			86
		16	F6066	10/10		46	51	3 27	–	9	0 9			98
R 3	1 2	1 ml/kg	Olive oil	5/5		23	25	2 97	1	2	0 6			
	1 2	32	F6066	1/5		–	–	–	1	–	1 0			–
	3–4	32	F6066	3/5		19	7	3 01[1]						54
	5 6	32	F6066	5/5		17	18	2 44	1	9	2 0			73
	7 8	32	F6066	3/5		14	12	3 22	1	5	2 0			54
R 5	1 2	2 ml/kg	Olive oil	7/8		33	48	3 39	1	4	0 7	3 0	4 6	
	1 2	16	F6066	0/8		–	–	–	–	–	–	–	–	–
	3–4	16	F6066	7/8		17	21	2 95	11	14	3 6	2 9	4 5	47
	5 6	16	F6066	5/8		19	20	3 34	5	2	1 4	2 9	4 8	48
	7 8	16	F6066	6/8	9	25	23	3 21	3	2	1 0	3 0	4 6	70
R 6	7 8 9 s c	2 ml/kg	0 9% NaCl	8/8	18	29	27	3 19	1	4	0 6	2 9	4 5	
	7 8 9 s c	25	Trypan blue	5/8		19	27	3 23	1	6	1 4	2 9	4 4	62
	7 8 9 s c	50	Trypan blue	4/8	8	17	11	3 30	–	–	–	2 7	4 5	49
	7 8 9 s c	100	Trypan blue	2/8		1	1	2 55	11[2]	2	6 5	2 5	–	3

[1] 8 small but living young (av weight 0 69 g) from one rat are not included in this figure
[2] 8 foetuses from one mother

Results

Effects on pregnancy and number of foetuses

F6066 Rats

A dose of 8 mg/kg of F6066 or less per day, on days 7, 8 and 9 after mating, produced no reduction in the number of living foetuses in rats (*table 14* R 1, R 2) When the mothers were given 16 mg/kg per day on days 7, 8 and 9 or 7-8 the number of foetuses was normal (R 2) or reduced (R 5) and when the daily dose given was 32 mg/kg, the number was reduced

Administration of F6066 in a dose of 64 mg/kg per day from day 7 until 5 days after birth, resulted in only 3 of 10 females giving birth to living offspring and on day 5 only 3 newborns from one litter were still alive (table 16) Similar treatment of 10 female animals but with 16 mg/kg resulted in the birth of 6 litters and in these, only 1 of the animals in one of the litters was found dead The growth of these newborns, the appearance of the sexual organs of those killed on day 5 and the weight of the sexual organs of animals killed when weaned were the same as in the control group The fertility of the animals at 3 months was also the same as in the control animals

F6066 Mice

In these animals the number of living foetuses of mothers treated with 8-64 mg/kg F6066 per day on days 6, 7 and 8 (M 1 and M 2 in table 15) was reduced Administration of 16 or 64 mg/kg on days 1-2, 3-4 or 5-6 resulted in impaired implantation or total abortion in most of the mothers, while the effect of F6066 diminished when given days 7-8 This is clear from M 3 and possibly from M 2 M 3 is a repetition of M 2 and was performed because the lack of fertility in the control group in M 2 made it difficult to draw any conclusions about the fertility of the groups that received the substance

F6103 Rats

The abortifacient action of F6103 was clearly demonstrated in the first two teratological experiments (R 7,R 8 in table 17) After 10 mg/kg, foetuses were only found in two of the five animals treated on days 7-8 After 3 3 mg/kg, foetuses were demonstrated in one animal from each of the two groups of six treated on days 5-6 and 7-8, respectively The uterus was fluid filled in some of the non pregnant animals which had received 10 mg/kg, in others, traces of a decidual reaction could be observed In the next three experiments (R 9, R 10 & R 11), F6103 was given later in pregnancy and in smaller doses This resulted in an increase in the number of foetuses in the treated animals Treatment with 0 05-0 2 mg/kg on days 10-19 resulted *in a halving of the* number of living foetuses, as compared with the controls

The pregnant animals were randomly distributed among experimental groups from day to day It took up to 4 weeks to collect sufficiently large groups of mice, and 1-2 weeks for rats

Treatment days were determined in relation to the day when spermatozoa were demonstrated or a vaginal plug was found (called day 0) The substance was generally given for 2 or 3 days Trypan blue was used as a positive control and was given subcutaneously in 0 9% NaCl (BECK & LLOYD 1964)

The female rats were killed on day 20 and the mice on day 18 A few animals were killed 1-2 days earlier These embryos were not included in the calculation of the average weight of the foetuses, since foetal weight increases by almost 100% on the last day of intrauterine life The mothers were anaesthetized with ether and bled The uterus was opened and the number of living and dead foetuses was noted as well as the lengths in millimetres of the dead foetuses Large foetuses were freed from the membranes, after which they were weighed and examined under a Leitz stereomicroscope (magnification × 10) The anogenital distance was measured with a measuring ocular and the abdominal and intra thoracic organs were removed and examined under the stereomicroscope

The foetuses were fixed in 80% ethyl alcohol They were then placed in 1 0 NaOH solution for 3 days and every day they were rinsed with water and placed in fresh solution For small foetuses 0 5% NaOH was used The bodies were then stained for 24 hours at room temperature with a mixture of 3 ml 0 1% alizarin dissolved in 1 0% NaOH + 100 ml 0 5% NaOH (the same amount of staining fluid per animal) The animals were then rinsed in water and placed in 20% glycerine When the embryos had sunk to the bottom they were transferred to 50% glycerine and then to 100% (Modified after BROCK & VON KREYBIG 1964 and LORKE 1963)

The bodies of the animals were now clear and translucent and the skeleton was bright red blue in colour The skeleton was examined under the stereo microscope for skeletal abnormalities The ribs, sternal bones and vertebrae were also counted

In one experiment (R 4) the female rats were allowed to give birth and keep their young The mothers were given either olive oil or F6066 dissolved in olive oil by stomach tube from the 7th day of pregnancy until 5 days after birth On day 5 some of the young were killed and the testicles ventral prostate and seminal vesicles or ovaries and uterus were removed and photographed so as to compare the size of these organs in the various groups Some of the new borns were killed when weaned and the sex organs weighed One male and one female from each litter were paired at 3 months of age with offspring from an other mother in the same group in order to check whether the animals were fertile Only half of the surviving control offspring were paired

The days on which the animals were treated with F6066 or F6103 and the doses used are given in tables 14 to 18

Results

Effects on pregnancy and number of foetuses

F6066 Rats

A dose of 8 mg/kg of F6066 or less per day, on days 7, 8 and 9 after mating, produced no reduction in the number of living foetuses in rats (table 14 R 1, R 2) When the mothers were given 16 mg/kg per day on days 7, 8 and 9 or 7 8 the number of foetuses was normal (R 2) or reduced (R 5) and when the daily dose given was 32 mg/kg, the number was reduced

Administration of F6066 in a dose of 64 mg/kg per day from day 7 until 5 days after birth, resulted in only 3 of 10 females giving birth to living offspring and on day 5 only 3 newborns from one litter were still alive (table 16) Similar treatment of 10 female animals but with 16 mg/kg resulted in the birth of 6 litters and in these, only 1 of the animals in one of the litters was found dead The growth of these newborns, the appearance of the sexual organs of those killed on day 5 and the weight of the sexual organs of animals killed when weaned were the same as in the control group The fertility of the animals at 3 months was also the same as in the control animals

F6066 Mice

In these animals the number of living foetuses of mothers treated with 8–64 mg/kg F6066 per day on days 6, 7 and 8 (M 1 and M 2 in table 15) was reduced Administration of 16 or 64 mg/kg on days 1 2, 3–4 or 5–6 resulted in impaired implantation or total abortion in most of the mothers, while the effect of F6066 diminished when given days 7–8 This is clear from M 3 and possibly from M 2 M 3 is a repetition of M 2 and was performed because the lack of fertility in the control group in M 2 made it difficult to draw any conclusions about the fertility of the groups that received the substance

F6103 Rats

The abortifacient action of F6103 was clearly demonstrated in the first two teratological experiments (R 7, R 8 in table 17) After 10 mg/kg, foetuses were only found in two of the five animals treated on days 7–8 After 3 3 mg/kg, foetuses were demonstrated in one animal from each of the two groups of six treated on days 5–6 and 7–8 respectively The uterus was fluid filled in some of the non pregnant animals which had received 10 mg/kg, in others, traces of a decidual reaction could be observed In the next three experiments (R 9, R 10 & R 11) F6103 was given later in pregnancy and in smaller doses This resulted in an increase in the number of foetuses in the treated animals Treatment with 0 05–0 2 mg/kg on days 10–19 resulted in a halving of the number of living foetuses, as compared with the controls

Table 16

Antifertile and abortive effects of F6066 given to pregnant rats by stomach tube from day 7 of pregnancy to day 5 after the birth.

Animal no		F6066	*No of young F_1*		*No ♂ and ♀ and av weight of offspring*						*Average weight of organs after weaning mg*					*No. of young: F_2 after remaining F_1♀*		Average weight at birth g
					killed day 5			when weaned										
			born	5 days later	♀	♂	weight g	♀	♂	weight g	ovaries	uterus	testes	prostate	sem. ves	born		
I	1	16 mg/kg	9	8	2	1	9 0	2	1	37	14	18	210	23	8 0	8	The test was discontinued 46 days after the males and females were placed together	6 6
I	2	"	–	–	–	–	–	–	–	–	–	–	–	–	–			
I	3	"	9	9	2	2	8 0	–	–	–	–	–	–	–	–			
I	4	"	11	11	2	3	7 4	2	2	39	15	23	169	23	8 0	pregn		
I	5	.		–	–	–	–	–	–	–	–	–	–	–	–			
I	6	"		–	–	–	–	–	–	–	–	–	–	–	–			
I	7	"	–	–	–	–	–	–	–	–	–	–	–	–	–			
I	8	"	13	13	2	3	10 2	–	2	43	–	–	183	19	6 5	12		5 1
I	9		1 (dead)	–	–	–	–	–	–	–	–	–	–	–	–			
I	10	"	8	8		3	10 7	1	2	43	17	22	179	24	8 0	10		4 8
II	1	(4 mg kg	–	–	–	–	–	–	–	–	–	–	–	–	–			

II 2		3 (1 dead)	7	1		15 0									7		7 7
II 3																	
II 4																	
II 5																	
II 6																	
II 7																	
II 8		1 (3 dead															
II 9		1 (dead)															
II 10																	
III 1	Ol e o l 1 ml kg	12	12	2	3	8 4	1		48	27	32						
III 2	″	13	13	3	3	7 7	3	2	40	18	24	185	25	6 5	8		6 4
III 3		6	6	1	1	12 0	1	1	36	13	13	143	17	9 0	pregn		
III 4		10	10	2	2	7 3	2	2	35	13	20	154	23	8 5	7		7 0
III 5	″	9	9	3	1	8 0	2		41	21	27				not p		
III 6		9	9		3	13 7	2	2	44	19	25	170	20	7 0			
III 7		10	10	1	3	8 5	2	3	43	17	20	198	23	7 0			
III 8		5	5		2	13 0	1	2	45	15	27	209	21	7 5			
III 9		7	7	2	1	9 0	1	1	40	14	22	170	19	8 0			
III 10																	

Table 17

Antifertility and teratogenic tests with F6103 in rats

Test	Substance adm by stom tube day	Dose/day mg/kg	Substance	No of pregn / no of rats	Young born before killed	Total no of liv foet per group ♀	Total no of liv foet per group ♂	Average weight of liv foet killed on day 20	Total no of dead foet per group >3mm	Total no of dead foet per group <3mm	Dead foet per pregn	Anogenital distance mm ♀	Anogenital distance mm ♂	Foet as %age of contr
R 7	1–2	1 ml/kg	Olive oil	4/5		21	16	3 40	1	–	0 3			
	1–2	10	F6103	0/5		–	–	–	–	–	–			–
	3–4	10	F6103	0/5		–	–	–	–	–	–			–
	5–6	10	F6103	0/5		–	–	–	–	–	–			–
	7–8	10	F6103	2/5		9	6	3 08	5	–	2 5			41
R 8	1–2	1 ml/kg	Olive oil	4/6	5	18	8	3 54	1	–	0 3	2 6	4 3	
	1–2	3 3	F6103	0/6		–	–	–	–	–	–	–	–	–
	3–4	3 3	F6103	0/6		–	–	–	–	–	–	–	–	–
	5–6	3 3	F6103	1/6		4	7	3 44	1	1	2 0	2 4	4 5	35
	7–8	3 3	F6103	1/6		5	2	3 25	2	–	2 0	2 6	4 3	23
R 9	7–8–9	1 ml/kg	Olive oil	6/10		36	24	3 30	1	7	1 3	2 9	4 6	
		0 5	F6103	5/10		22	20	3 30	–	9	1 8	2 9	4 7	70
		1 0	F6103	3/10		9	18	3 46	–	3	1 0	3 0	4 6	45
		2 0	F6103	6/10		16	36	3 32	13	7	3 3	2 8	4 5	87
R 10	10–11–12	1 ml/kg	Olive oil	7/10		40	35	3 30	2	9	1 6	2 9	4 6	
		1 0	F6103	8/10		23	27	2 97	35	4	4 9	2 8	4 5	67
		2 0	F6103	4/10		14	12	3 06	15	2	4 3	2 7	4 5	35
		4 0	F6103	6/10		5	11	2 65	46	5	8 5	2 7	4 2	21
R 11	10–19	1 ml/kg	Olive oil	8/10		48	46	3 21	–	–	–	3 1	4 8	
		0 05	F6103	6/10		31	27	3 26	2	6	1 3	3 0	4 7	62
		0 1	F6103	6/10		26	26	3 29	4	5	1 5	3 0	4 9	55
		0 2	F6103	6/10		21	22	3 39	5	4	1 5	3 1	4 8	46

Table 18

Antifertility and teratogenic tests with F6103 in mice

Test	Substance adm by stom tube day	Dose/day mg/kg	Substance	No of pregn / no of rats	Young born before killed	Total no of liv foet per group		Average weight of liv foet killed on day 20	*Total no of* dead foet per group		*Dead* foet per pregn	Anogenital distance mm		Foet as %age of contr
						♀	♂		>3mm	<3mm		♀	♂	
M 6	6–7–8	10 ml/kg	Olive oil	6/8		28	26	1.24	1	3	0.7	2.3	3.3	
		2.5	F6103	1/8		2	3	1.09	4	1	5.0	2.0	3.0	9
		5	F6103	1/8	6	–	–	–	–	–	–	–	–	11
		10	F6103	0/8		–	–	–	–	–	–	–	–	–
M 7	6–7–8	10 ml/kg	Olive oil	7/9		36	27	1.30	–	4	0.6	2.1	3.2	
		0.5	F6103	3/9		11	14	1.31	–	2	0.7	2.3	3.2	40
		1.0	F6103	3/9		13	13	1.32	3	2	1.7	2.2	3.2	41
		2.0	F6103	0/9		–	–	–	–	–	–	–	–	–
M 8	9–10–11	10 ml/kg	Olive oil	6/10		18	24	1.26	1	1	0.3	2.1	2.8	
		1.0	F6103	7/10		25	22	1.27	17	2	2.7	2.1	3.2	112
		2.0	F6103	5/10		15	8	1.22	13	6	3.8	2.1	2.9	55
		4.0	F6103	5/10		12	10	1.16	10	6	3.2	2.0	2.9	52
M 9	9–10–11	10 ml/kg	Olive oil	8/10	12	18	26	1.34	1	1	0.3	2.2	3.1	
		0.5	F6103	4/10		10	16	1.32	3	2	1.3	2.3	3.1	46
		1.0	F6103	3/10		12	10	1.45	2	2	1.3	2.1	3.3	39
		2.0	F6103	3/10		11	12	1.28	1	3	1.3	2.2	3.0	41
M 10	9–17	10 ml/kg	Olive oil	8/10	21	28	27	1.35	1	1	0.3	2.3	3.2	
		0.025	F6103	8/10		27	27	1.35	–	11	1.4	2.1	3.2	71
		0.05	F6103	6/10		24	35	1.32	1	3	0.7	2.2	3.2	78
		0.1	F6103	6/10		23	17	1.34	1	7	1.3	2.2	3.1	53

Table 17

Antifertility and teratogenic tests with *F6103* in rats

Test	Substance adm by stom tube day	Dose/day mg/kg	Substance	No of pregn / no of rats	Young born before killed	Total no of liv foet per group		Average weight of liv foet killed on day 20	Total no of dead foet per group		Dead foet per pregn	Anogenital distance mm		Foet as %age of contr
						♀	♂		>3mm	<3mm		♀	♂	
R 7	1–2	1 ml/kg	Olive oil	4/5		21	16	3 40	1	–	0 3			
	1–2	10	F6103	0/5		–	–	–	–	–	–			–
	3–4	10	F6103	0/5		–	–	–	–	–	–			–
	5–6	10	F6103	0/5		–	–	–	–	–	–			–
	7–8	10	F6103	2/5		9	6	3 08	5	–	2 5			41
R 8	1–2	1 ml/kg	Olive oil	4/6	5	18	8	3 54	1	–	0 3	2 6	4 3	
	1–2	3 3	F6103	0/6		–	–	–	–	–	–	–	–	–
	3–4	3 3	F6103	0/6		–	–	–	–	–	–	–	–	–
	5–6	3 3	F6103	1/6		4	7	3 44	1	1	2 0	2 4	4 5	35
	7–8	3 3	F6103	1/6		5	2	3 25	2	–	2 0	2 6	4 3	23
R 9	7–8–9	1 ml/kg	Olive oil	6/10		36	24	3 30	1	7	1 3	2 9	4 6	
		0 5	F6103	5/10		22	20	3 30	–	9	1 8	2 9	4 7	70
		1 0	F6103	3/10		9	18	3 46	–	3	1 0	3 0	4 6	45
		2 0	F6103	6/10		16	36	3 32	13	7	3 3	2 8	4 5	87
R 10	10–11–12	1 ml/kg	Olive oil	7/10		40	35	3 30	2	9	1 6	2 9	4 6	
		1 0	F6103	8/10		23	27	2 97	35	4	4 9	2 8	4 5	67
		2 0	F6103	4/10		14	12	3 06	15	2	4 3	2 7	4 5	35
		4 0	F6103	6/10		5	11	2 65	46	5	8 5	2 7	4 2	21
R 11	10 19	1 ml/kg	Olive oil	8/10		48	46	3 21	–	–	–	3 1	4 8	
		0 05	F6103	6/10		31	27	3 26	2	6	1 3	3 0	4 7	62
		0 1	F6103	6/10		26	26	3 29	4	5	1 5	3 0	4 9	55
		0 2	F6103	6/10		21	22	3 39	5	4	1 5	3 1	4 8	46

Some of the animals classified as ' not pregnant ' had, in fact, been pregnant at some stage, since the uterine wall was nodular in some areas Since the diagnosis of pregnancy was made merely by visual inspection, it must be understood to be a subjective impression, especially where treatment was started early in pregnancy

Trypan blue Rats and Mice

In rats a dose dependent decrease in the number of pregnant animals, the number of living foetuses and the mean foetal weight was seen In both rats and mice Trypan blue tended to increase the number of foetal deaths (table 14 R 6 and table 15 M 1)

Malformations

In the present investigation, neither F6066 nor F6103 seemed to be teratogenic in our strains of rats and mice although in 3 cases fused ribs were seen after F6103 dosing Skeletal development was retarded in 2 small rat foetuses, each weighing 1 84 grams from F6103 treated mothers, e g the sternal bones were not stained by alizarin The frequency of malformations increased after Trypan blue administration in mice to 4 4% from zero level in the control group The anogenital distance did not alter after F6066 or F6103 as compared with controls if the treated foetuses were of normal weight

The number and types of gross malformations after Trypan blue, F6066, F6103 and olive oil are given in table 19 Small differences in the maturity of the skeleton e g number of ribs and breast bones that stained with alizarin, are not included because this varied in all the groups

Comments

As shown on pages 38 to 47 F6066 and F6103 are abortifacient in rats and mice when given in sufficient doses during pregnancy During this study we wanted to use as large doses of F6066 and F6103 as possible, since if such large doses produced no malformations small doses could be assumed not to do so either We therefore tried to regulate the doses in such a way as to cause abortion in some of the animals but not in all of them As shown above, in certain cases we used doses which produced total abortion in most of the experimental animals This was partly because the abortion and teratological experiments were carried out in parallel

In those cases where a dose response relation was established in this experiment, the dosage period was not the same as in the abortion experiments Thus a direct comparison of the ED50s for abortion in the two types of experiment

F6103 Mice

The abortifacient effect of F6103 was also very prominent in the first of these experiments (M 6 in table 18) Even 2 mg/kg F6103 on days 6, 7 and 8 (M 7) produced total abortion in all animals treated In both M 8 and M 9, 1 0 and 2 0 mg/kg F6103 were given on days 9, 10 and 11 The number of young was not reduced by 1 0 mg/kg in M 8 but was halved by the same dose in M 9 0 025–0 1 mg/kg given on days 9–17 reduced the number of living foetuses by 30–50%

Effects on weight of the foetuses and on the frequency of dead foetuses

F6066 and F6103 Rats and Mice

On 3 occasions (R 3, M 2, M 5) we found that foetuses from mothers treated with F6066 were substantially smaller than those of control foetuses The foetuses were living and normally developed, as far as we could see with our technique Eight rat foetuses with an average weight of 0 69 g compared with normally more than 3 g, were found in the same mother in R 3 The 8 foetuses were examined at the Embryological Institute in Lund by Professor B KÄLLÉN, who found them somewhat premature, but showing no signs of malformation Examination of the 6 small mouse foetuses after careful staining with alizarin showed normal maturation and no skeletal changes Apart from the low weight of these few animals, no difference in mean weight and weight distribution was found between the F6066 and control groups

Only one rat foetus with a body weight of under 1 gram was found after F6103 treatment This foetus was in a uterus together with 11 dead foetuses of over 3 mm from a rat given 4 mg/kg on days 10, 11 and 12 The mean weight of the foetuses was reduced in some groups from experiments R 7, R 8 and R 10 Even in the mouse experiments, the mean foetal weight was reduced after F6103 treatment in some cases (M 6, M 8 and M 9) In two animals (after 0 5 mg/kg on days 9, 10 and 11 and after 0 025 mg/kg on days 9–17), a living mouse foetus was found which was markedly smaller than the other foetuses in the litter, the smallest weighed only 0 76 grams

In both mice and rats, F6066 and F6103 increased the number of dead foetuses per pregnant mother In rats given F6103, for example, the number of dead foetuses per mother varied between 1 0 and 8 5, whereas in the appropriate control groups the frequency varied between 0 and 1 6 The highest frequency was observed after treatment with 1–4 mg/kg on days 10, 11 and 12 A tenth of this dose given on days 10–19 resulted in a reduced number of living foetuses without a notable increase in the number of dead ones An increased frequency of dead foetuses per mother was found in all groups where the mean foetal weight was lower than in the controls

is not possible, although the order of size of the doses used to produce abortion was the same

F6066 or F6103 did not demonstrably increase the frequency of malformations In our experiments Trypan blue produced an increase in the number of malformed foetuses from 0 0% in control mice to 4 4%, while the frequency in rats was nearly unchanged BECK & LLOYD (1964) reported 5–20% malformed rat foetuses following corresponding doses of Trypan blue given in a single dose 8½ days after conception Although they do not state whether the day on which the animals were mated was called day 0 or day 1, the animals were treated during approximately the same period as that used in the present investigation, and the frequency of malformations they reported was substantially higher BEAUDOIN & ROBERTS (1965) suggest that the effect of Trypan blue should be sought in serum protein changes in the mother of the foetus, but obtained no experimental evidence to support their assumption on injection of serum protein fractions from non pregnant animals and pregnant rats treated with Trypan blue In a discussion between GILLMAN and BECK (1965) the possibility of changes in macromolecules was pointed out, but decreased transport of oxygen in the placenta and changes in the placental enzymes were also mentioned as possible precipitating factors The reason for the weak effect in our animals is obscure but it may have been due to the use of different strains or to differences in the quality of the Trypan blue used It can hardly be ascribed to inaccurate or insufficient examination of the foetuses, because the frequency (0 9%) of severe spontaneous malformations in the control group of rats appeared normal

Against the background of a frequency of 0 9% in our rat strain, it was unexpected that not a single malformation was observed in 450 control mouse foetuses

NISHIHARA (1958) showed that oestrogens (oestradiol and oestrone) increase the foetal mortality and the frequency of cleft palate if the substance is injected into mice between the 11th and 16th day of pregnancy In none of our foetuses was a cleft palate observed in the experiment (R 4), in which we gave F6066 late in pregnancy or in the experiments where F6103 was given during days 10–19 or 9–17

Triparanol (Mer 29) stunts the growth of young male rats, it causes xerophtalmia and reduces the fertility of both males and females, if given to their mothers during pregnancy (WEXLER 1964, ROUX & DUPUIS 1961) A single dose is sufficient to cause this effect, which can be counteracted by simultaneous administration of ACTH The effect is ascribed to the anticholesterolic properties of Triparanol The effect of F6066 and F6103 on the serum cholesterol is much smaller than that of triparanol (LARSSON, personal communication)

Very small but well-developed foetuses were found in 3 mothers treated with F6066 but not in the control group The mean foetal weight for the F6103

Table 19

Type and number of major malformations in living mice and rat foetuses treated with Trypan blue, F6066, F6103 and Olive oil

Substance	Dosed day	Dose mg/kg	Test no	Type of malformation	No of foetuses mice/rats	Mal formations mice/rats
F6066	7–8–9	8	R 1	Small parietal and temporal bones without exencephalon		
	7–8–9	8	R 2	No right pubic bone		
	7–8 9	16	R 2	One ear large and malformed		
	6–7–8	8	M 4	3 sternal bones unsymmetrical		
	7–8	16	M 5	4 sternal bones unsymmetrical	150/536	2/3 (1 3/0 6 %)
F6103	6–7–8	0 5	M 7	Partial anencephalon		
	6–7–8	0 5	M 7	2 ribs originating from same double vertebra on both sides		
	9–10–11	0 5	M 9	2 ribs originating from same double vertebra on both sides		
	9–10–11	2 0	M 9	2 fused ribs on one side and three on the other		
	10–19	0 05	R 11	Partial anencephalon, skeleton less developed than in siblings		
	9–17	0 1	M 10	Sternal bones arranged in a zig zag pattern	372/399	5/1 (1 3/0 3 %)
Trypan blue	6–7–8	100	M 1	Exencephalon, malformed eye		
	6–7–8	100	M 1	Exencephalon		
	7–8–9	100	R 6	No tail, anus or lumbar and sacral vertebrae	45/76	2/1 (4 4/1 3 %)
Control	7–8 9		R 2	Exencephalon } same mother		
	7–8 9		R 2	Exencephalon } same mother		
	1–2		R 5	No right or left pubic bone and 5 sternal bones missing		
	7–8 9		R 6	One ear large and malformed		
	10–19		R 11	Only two sternal bones fewer caudal vertebrae stained than usual		
	10 19		R 11	Twelfth rib on right side unossified	450/634	0/6 (0/0 9 %)

Antagonism of injected progesterone's effects in the Clauberg test

Introduction

The history of progesterone is related in CORNER's (1946) book, "The Hormones in Human Reproduction", which includes a description of CORNER & ALLEN's demonstration of a factor in ovarian extract which could produce a pregnancy like endometrium in pregnant, oophorectomized rabbits. Ovulation was established at laparotomy 18 hours after mating by examining the ovaries for ruptured follicles and oophorectomy was performed at the same time. The rabbits were thereafter given injections of the extract for five days and sacrificed on the sixth. A positive gestagen effect was recorded only if the uterus exhibited full proliferation equivalent to an undisturbed pregnancy of the same length.

CLAUBERG (1930) modified the experiment by using immature rabbits weighing 600–800 grams. The ovaries of such animals have not usually produced sufficient oestrogen to make the uterus sensible to progesterone but this is achieved by eight daily injections of oestrogens before progesterone administration. The effect of injected progesterone is determined histologically. The method has been used for biological standardization of progesterone. MCPHAIL (1934) graded the endometrial changes in six degrees, but both CLAUBERG's and MCPHAIL's methods can only be regarded as semiquantitative.

The effect of F6066 injected in a modified CLAUBERG test has been briefly described by EINER JENSEN (1965) and HANNGREN *et al.* (1965b).

Method

A modified CLAUBERG (1930) test was carried out on immature female rabbits (600–800 grams).

After priming with 0.5 µg EB for 7 days, combinations of 0.2 mg/kg progesterone and various doses of the test substances were injected subcutaneously in oil for a further 7 days. The doses of F6066, F6103, EB and DES are shown in table 20. Usually two animals per dose were used. In certain experiments with F6066 the progesterone dose was increased and in other cases the test substance was given only on days 13 and 14.

On the 15th day, the animals were killed, the weight of the uterus was determined and a piece of uterus was fixed in Bouin's solution. The fixed tissue was sectioned at 5–10 µ and stained with haemalum and eosin or van Gieson

treated groups decreased in some cases and was unchanged in others A few very small foetuses were also seen after F6103 administration This poor growth can probably be attributed to an inhibiting effect of the substances on the decidua directly and/or indirectly via the corpora lutea Administration of F6066 or F6103 for 2–3 days can therefore lead to an insufficient decidua-formation and consequently failure of implantation or, if the substances are administered later in pregnancy, to an insufficient placental development An "almost" abortive dose need not mean foetal death, but can lead to decreased nutrition These small foetuses, together with the increased number of dead or resorbed foetuses seen even in uteri which also contained living foetuses, indicate that the effect of F6066 and F6103 on gestation is not of the "all or none" type

The lower weight of the foetuses may also be due to delayed implantation *When rats were ovariectomized on day 2 (conception = day 0) and then given* progesterone daily, the blastocysts did not become implanted but implantation did occur when 0 5 µg oestrone was injected (MAYER 1959) SCHLOUG & MEYER (1965) showed that antioestrogens can delay implantation, but this effect has apparently not been described in animals given larger doses of oestrogens or antigestagens

As described on page 74, only traces of ^{14}C F6066 or its radioactive metabolites pass the placental barrier after intravenous administration to pregnant mice A direct effect of the substance on the foetus therefore appears unlikely

The very small foetuses seen after F6066 treatment, which were sometimes only one third the normal size, cannot be regarded as "congenital runts" (MCLAREN & MICHIE 1960), since they do not occur in association with normally sized foetuses in the same litter The small foetuses seen after F6103 may be runts, as most of them were seen together with living foetuses of normal weight, but on the other hand, they also always occurred in groups with an increased frequency of dead foetuses

The results previously described for rabbits and dogs further support the conclusion that F6103 is not teratogenic in animal experiments

Table 20 cont

F6103		+ Progesterone		Wet wt uterus	Histological appearance	Body wt at sacrifice
mg/kg	day	mg/kg	day	g		kg
0 40	"	"	"	5 3	++	1 2
0 40	"	"	"	6 3	++	1 0
0 50	13 14	"	"	3 3	+	0 77
0 80	8–14	"		6 1	++	1 6
0 80	"	"	"	5 4	++	1 5
DES µg/kg		+ Progesterone				
0 20	8 14	0 20	8 14	2 2	0	1 3
0 20	"	"	"	2 4	0	1 1
1 0	"	"	"	3 3	+	1 1
2 0	13 14	"	"	2 8	0	1 2
2 0	"		"	2 2	0	1 3
2 0	11–14	"	"	4 8	+	1 2
2 0	"	"	"	3 8	+	1 3
2 0	8–14	"	"	3 9	++	1 4
2 0		"		5 1	+	1 1
5 0	13–14	"	"	2 2	++	1 2
5 0	"	"	"	2 2	++	0 97
F6066 mg/kg		+ Progesterone				
0 50	8 14	0 20	8 14	2 6	+	1 6
0 50		"	"	2 7	+(+)	1 6
1 0		"	"	3 1	+	1 2
1 0	"	"	"	4 3	+	1 3
1 0	"	"	"	5 1	+(+)	1 3
1 0		"	"	4 3	+	1 5
1 0		"	"	3 2	++	1 1
1 0	"	"	"	5 8	+	1 5
1 0	"	0 40	"	3 8	(+)	1 2
1 0	"	0 40	"	5 1	0	1 5
1 0	"	0 80	"	5 2	0	1 5
1 0	"	0 80	"	3 9	+	1 5
2 0	"	0 20	"	4 1	++	1 2
2 0	"	"	"	4 9	++	1 4
2 0	"	"	"	5 8	+(+)	1 3
1 0[1]	"	"	"	1 9	(+)	1 6
1 0[1]	"	"	"	3 0	(+)	1 5
5 0	11 14	"	"	7 4	++	1 1
5 0	13–14	"	"	5 1	+	1 2

The histological appearance of the endometrium was classified as follows an endometrium corresponding to *pure* oestrogen influence (as by 0 5 μg EB daily for 14 days) was *recorded* as ++, a gestagen dominated endometrium was recorded as 0, and an *intermediate state as* + *FD50* for inhibition was de fined as that dose which produced a + endometrium

Results

The uterus increased in weight after daily injection of 0 5 μg EB for 7 or 14 days There was proliferation of both endometrial glands and stroma, and

Table 20

Anti Clauberg test on *immature* rabbits The animals were given s c injections of EB 0 5 γ/kg on days 1–7 *and* of the following combinations on days 8 14 and were sacrificed on day 15

F6103		+ Progesterone		Wet wt uterus	Histological appearance	Body wt at sacrifice
mg/kg	day	mg/kg	day	g		kg
0 0125	8–14	0 20	8 14	4 7	0	1 4
0 0125				3 7	(+)	0 96
0 015				2 5	(+)	1 5
0 015				4 4	(+)	1 8
0 020	"			4 8	(+)	1 3
0 020				4 1	0	1 4
0 025		"		3 0	+	1 2
0 025				4 7	+	1 3
0 025				3 5	+	1 1
0 025				2 8		1 3
0 030				3 8	++	1 3
0 030				4 9	+	1 7
0 045				4 0	++	1 3
0 045				4 7	+(+)	1 4
0 045				3 8	+	1 3
0 045				3 1	+	1 0
0 050				3 6	+(+)	1 0
0 050				3 2	+(+)	0 86
0 050				3 6	++	0 99
0 050				4 0	(+)	1 3
0 080				7 0	++	1 4
0 080			"	6 0	++	1 5
0 10				5 1	++	1 0
0 10				4 8	++	1 2
0 10		"	"	6 7	++	1 4
0 10		"		4 5	++	1 1
0 20		"	"	5 4	++	1 5
0 20			"	5 5	++	1 5

to overcome the inhibitory effect of 1 mg/kg F6066 by increasing the progesterone dose from 0 2 mg/kg to 0 4 or 0 8 mg/kg, but this experiment was carried out in only a few animals

Comments

In this test F6066 and F6103 were able to antagonize the effect of injected progesterone on a target organ, the uterus This antiprogesterone effect may be regarded as an antifertility factor, since it is improbable that implantation can take place after such pronounced histological changes in the endometrium

Even DES and oestradiol benzoate had an antiprogesterone effect in this test As shown in table 20, uterine weight increases as the antiprogesterone effect appears with increasing doses of all four substances In an unpublished experiment, the author has shown that the dose response curves for uterine weight after F6066 and oestradiol benzoate run parallel at the dose levels used here and that the ratio between wet and dry weight is about seven for both substances The oestrogenic effect was also manifested by oedema formation and an increase in the number of eosinophils in the uterus The antiprogesterone effect of F6066 and F6103 is thus probably related to the conventionally defined oestrogenic effect

When the progesterone dose was increased from 0 2 to 0 4 and 0 8 mg/kg in animals given 1 mg/kg F6066, the histological picture approached that usual for a secretory endometrium This suggests that it is the relation between oestrogen and gestagen influence which is important, whilst the absolute dose required to produce changes is flexible

Table 20 cont

EB		+ Progesterone		Wet wt uterus	Histological appearance	Body wt. at sacrifice
µg/kg	day	mg/kg	day	g		kg
0 50	1–14	0 20	8–14	2 9	+	1 3
0 50	"	"	"	2 8	(+)	1 1
2 0	"	"	"	4 7	++	1 1
2 0	"	,	"	4 1	++	1 3
		Progesterone				
		0 10	8–14	1 6	+(+)	1 5
		,	"	0 9	+	1 0
		0 40		3 4	0	1 4
		"	,	2 8	0	1 0
		0 20	"	1 8[2])	0[2])	1 3[2])
EB		Olive oil				
0 50	1–14		8–14	1 3	++	1 0
0 50	1–7		"	1 2	++	1 5
0 50	"		"	1 2	++	1 0

[1]) Per os in oil [2]) Mean for 20 animals
Classification
++ histological picture corresponding to pure EB influence
0 histological picture corresponding to pure progesterone influence
+ partially inhibited progesterone effect

neutrophils, eosinophils and oedema were often observed in the stroma and myometrium

The development of the uterus after progesterone administration was as earlier described The endometrium proliferated and folded, forming a structure which in cross-section was reminiscent of the contours of a tree The endometrial cells and the cells of the uterine glands exhibited a secretory appearance

If F6066, F6103, EB or DES were given together with progesterone, there was a dose-dependent inhibition of the endometrium's secretory phase and the development of the tree-like formation ED50 for F6066 was estimated to be 1 mg/kg, for F6103 0 03 mg/kg, for EB 0 7 µg/kg and for DES 3 µg/kg (table 20) When F6066 was given only on days 13 and 14, there was regression of the secretory endometrium which had already been built up It appeared possible

−70°C in a mixture of acetone and carbon dioxide The intervals chosen were 5 minutes, 20 minutes, 1 hour, 4 hours and 24 hours

The substance was given subcutaneously in 3 animals (1 male, 1 non-pregnant and 1 pregnant female), all of which were sacrificed 20 minutes after the injection

Four pregnant mice and one male mouse were injected intravenously with F6066, prepared in the same way as F6060, in a dose of 1 mg per mouse or 12 μc The pregnant mice were sacrificed 5 minutes, 20 minutes, 1 hour and 24 hours after injection, and the male mouse 4 hours after injection

The frozen mice were transferred to a refrigerated room at −10° C and sagittal sections of 20 μ were taken through the whole animals at different levels Apposition autoradiograms were made by pressing the freeze-dried sections against a Gevaert Structurix X ray film (ULLBERG 1958) The exposure time was 3 months for F6060 and 2 months for F6066

Results

The two substances behaved differently in that the blood concentration remained higher after the injection of F6060 than after the injection of the diacetate, F6066

Accumulation was observed in the same organs for the two compounds but the diacetate showed a more distinct organ localization as compared with the surrounding tissue

The most remarkable finding with both substances was a very distinct and selective accumulation in the copora lutea A slight accumulation was also observed in the ovarian follicles and stroma of non pregnant mice, the inter-

Liver Corpora lutea

Fig 4 Autoradiagram from a pregnant mouse 20 minutes after intravenous injection of ^{14}C F6066 Note high radioactivity in corpora lutea and liver (Structurix, Gevaert ×0 9)

Distribution Pattern of ^{14}C-F6066 and ^{14}C-F6060

Introduction

The distribution of F6066 and F6060 has been studied in the mouse by a whole body autoradiographic method in cooperation with HANNGREN and ULLBERG (Hanngren et al 1965 a)

Methods

Preparation of bis-(p-hydroxyphenyl)cyclohexylidene-^{14}C methane
(KARLSSON & SUNDBECK, pers comn)

The Grignard reagent from cyclohexyl chloride was allowed to react with ^{14}C-carbon dioxide to form cyclohexane carboxylic-^{14}C acid By reaction with diazomethane the methyl ester was formed

This methyl ester was allowed to react with the Grignard reagent from p-bromoanisol and the resultant product was demethylated with alkali at 200°C to form bis(p-hydroxyphenyl)cyclohexylidene-^{14}C methane The specific activity was 10 μc/mg

Preparation of bis(p-acetoxyphenyl)cyclohexylidene-^{14}C methane

The phenol was treated with acetic anhydride and a trace of sulphoric acid, thus forming bis(p acetoxyphenyl)-^{14}C methane The specific activity was 12 μc/mg

Autoradiographic studies

Twenty-three adult white mice including 16 females (10 of which were in a late stage of gestation) and 7 males were used The mean weight was about 20 g for male and non-pregnant female mice and about 35 g for pregnant mice The radioactive compound F6060 was dissolved in a minimal volume of absolute alcohol, mixed with a 5% aqueous Tween 80 solution, and injected intravenously in 15 animals Each animal received 0 2 ml, containing 0 8 mg of the compound (8 μc)

The animals were killed in groups of three (1 male, 1 non-pregnant female and 1 pregnant female) at intervals after injection by rapid freezing to about

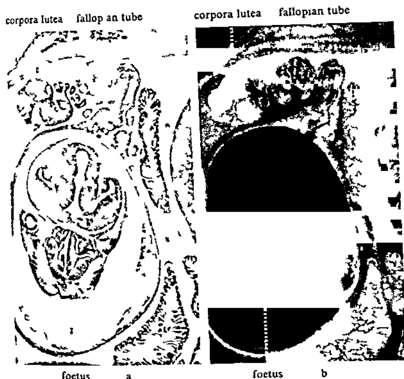


Fig 5 Autod agram (b) showing the distribution of ^{14}C-F6060 in ovary and pregnant uterus 5 minutes after intravenous inject on Correspond ng tissue section (a) s stained w th haemalum-eos n Note the very h gh concentration in the corpora lutea and the very low concentration in the endometr um No act vity in the foetus (Structurix Gevaert. ×5)

The male genital organs The interstitial tissue of the testis showed a rather rapid and pronounced accumulation lasting for 1 hour Four hours after the injection no activity was seen A slight accumulation was also seen in the seminal vesicles and in Cowper s glands No concentration was observed in the prostate

The female genital organs In the ovary of the pregnant mice the corpora lutea already showed very high activity after 5 minutes (fig 5) This accumulation was also marked after 20 minutes (fig 4) and then progressively decreased but was still visible after 24 hours (fig 7) In the non pregnant mice some corpora lutea had a very high activity and some a very low activity The growing follicles showed activity in the walls after 20 minutes (fig 6a) and in the fluid after 1 hour (fig 6b) The interstitial cells in the ovary of non pregnant mice had a rather high activity

The endometrium and the wall of the fallopian tube showed only a slight accumulation (fig 5) The placenta had an activity slightly higher than the

stital tissue of the testes, and in the foetal membranes, especially in the yolk sac epithelium

The main excretory routes were through the kidneys, especially soon after the injection, and through the liver to the intestinal tract Excretion was also observed through the gastric but not through the intestinal epithelium

The distribution in various tissues at different intervals after administration of ^{14}C-F6060 is described below in more detail As stated above, the findings made in the F6066 investigation were very similar

Skeleton The compact bone showed no radioactivity The concentration in the bone marrow parallelled that in the blood

Musculature The skeletal muscles showed a fairly low activity which never reached that of the blood The concentration in the myocardium, however, was always somewhat higher than that in the skeletal muscles, and was higher in the pregnant than in the non pregnant and male mice

Connective tissues A relative increase in the radioactivity of connective tissues as compared with blood and muscle was seen up to one hour after ad ministration The cartilage showed a low concentration of radioactivity

Lymphatic tissue Lymph nodes and thymus showed almost no activity The red pulp of the spleen showed the same activity as the blood and bone marrow

Respiratory organs No accumulation could be observed, the organs following the concentration in the blood during the whole observation period

Digestive tract The mucosal glands at the base of the tongue and the salivary glands showed a rather high activity up to 1 hour after administration In the gastric mucosa there was moderate accumulation in the basal portions of the glands Activity in the intestinal lumen gradually increased due to excretion via the bile There were no signs of excretion through the intestinal mucosa

Liver The liver showed a high and long lasting radioactivity (fig 4) The activity increased up to 1 hour and then slowly decreased Five minutes after injection, the gall bladder already contained high radioactivity

Pancreas A fairly high activity was seen from 5 to 60 minutes after injection The activity at 20 minutes was almost as high as that of the liver and kidney

Kidney A high activity was seen from 5 to 60 minutes after injection The activity at 4 and 24 hours was low

Central nervous system A fairly high concentration in the grey matter was seen from 5 to 60 minutes after injection Plexus chorioideus showed rapid and transient activity The eye showed some activity in the retina up to 1 hour after the injection

Adrenals There was a rapid and rather high accumulation of activity in the cortex, but not in the medulla Some activity was also seen after 24 hours

Hypophysis A fairly high activity was observed from 5 minutes up to 4 hours after injection

Thyroid Almost no activity was seen



Fig 7 Autoradiogram showing the distribution of ^{14}C F6060 in a pregnant mouse 24 hours after intravenous injection Note high radioactivity in the visceral yolk sac epithelium Radioactivity is seen in 5 corpora lutea and in 3 placentae while the foetuses show no radioactivity (Structurix Gevaert ×2 5)

lutea and yolk sac epithelium The substances seem to be excreted during the first hour by the kidney and liver and through the gastric wall, and later mainly by the liver The very slight placental passage indicates that the direct effect on the foetus is relatively very small

Apart from the endocrine organs the only ones showing accumulation were the liver kidney salivary glands and pancreas The concentration in the salivary glands as well as in the pancreas may only indicate excretion

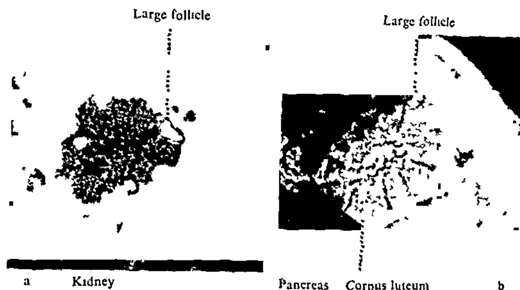


Fig 6 Autoradiogram showing the distribution of ^{14}C F6060 in the ovary, kidney and pancreas of non pregnant mice after intravenous injection Note high radioactivity in the *interstitial* cells of the ovary Growing follicles show radioactivity in the walls after 20 minutes (a) and in the *liquid* after 1 hour (b) (Structurix, Gevaert ×5)

blood from 5 minutes to 1 hour after the injection The visceral yolk sac epithelium, however, showed very high activity similar to that of corpora lutea but appearing later From 4 hours and onwards the concentration in the visceral yolk sac was the highest in the whole body (fig 7)

Foetuses A very small amount of radioactivity passed from the mother to the foetus In the foetuses the highest activity was seen in the adrenal cortex and the excretory pathways

Comments

The rapid, marked and selective accumulation of the radioactive substances in corpora lutea, is the most remarkable finding and is dealt with in some detail in the general discussion (page 83)

In the autoradiographs a relatively small and transient concentration was found in the hypophysis As many compounds, even those without endocrine effects, show high concentration in the hypophysis, it is difficult to evaluate the significance of this localization

The reduction in the size of the prostate which has been shown following the administration of F6066 to animals (page 6) cannot be correlated with an accumulation in this organ *The accumulation found in the interstitial tissue of the testes suggests a possible interference with testosterone formation*

A rather rapid disappearance from the organs was observed After 24 hours the radioactivity had disappeared from all organs except the liver, corpora

The antifertility effect of the twice weekly injections of F6103 or DES in rats shows that these substances can interrupt pregnancy None of the interrupted pregnancies can have progressed more than 6 days. Since the days of treatment were not coordinated with the day of mating, these results suggest that the antifertility effect can operate during the entire first week of pregnancy, but it is not possible to devine whether F6103 or DES are more active on certain days than on others

In the anticlauberg test, both conventional oestrogens and the F substances fulfil the definition of antigestagens An important question is therefore whether the F substances are antifertile merely because of their oestrogenic properties or whether they also have, for example, antiluteotrophic properties

If the effects of the F substances are based upon their oestrogenic properties, it should be reasonable to compare the strength of these with that of the other properties found here, even if the type of ratio calculation involved is affected by such sources of error as differences in the periods of treatment, castrated animals contra pregnant animals, and so forth

The upper rows of table 21 show the ratios between the ED50s for abortion on days 3–4 or 11–12 in pregnant rats and vaginal oestrogenicity in castrated rats for ethynyl oestradiol, dienoestrol, DES, EB, F6066 and F6103 The lower rows of the same table show similar ratios based upon the ED50s for abortion on various days of mouse pregnancy and the uterotrophic effect in mice for all these substances Thereafter, ratios are presented between the antifertility ED50s for DES, F6066 and F6103 given to rats seven times or twice weekly and the vaginotrophic effect in rats In addition, a ratio has been calculated between the ED50s for abortion on days 11 12 and 3–4

The methods used to determine oestrogenicity have been described previously (Einer Jensen 1965) When judging the table, it should be borne in mind that subcutaneous administration was used in the tests for determining oestrogenicity whilst oral administration was used in most of the other tests (with the exception of EB)

It is clear from the table that the ratio *abortion days 3–4/oestrogenicity* varies considerably from one substance to another in both rat and mouse experiments The weak abortifacient effect of ethynyl oestradiol in rats as compared with its oestrogenicity is striking The ratio *abortion days 11 12/oestrogenicity* also reveals variations between the oestrogenicity of the substances used and their abortifacient effect

The ratios calculated between *the ED50s for abortion on days 11–12 and 3–4* show that the abortifacient effect of F6103 can only partly be explained by the substance's oestrogenic properties When given to mice on days 11–12, the effective dose of F6103 was seven times greater than on days 3–4, the ED50s for both EB and DES were 20 times greater In the equivalent rat experiments, F6103 was effective in double the dose, whilst DES and dienoestrol had to be given in 3–10 times greater amounts EB, given subcutaneously, was found to

Discussion

The terms "antigestagen" and "antiluteotrophic" will be used in the following discussion A substance can be defined as a gestagen if it can provoke the formation of a secretory endometrium in oestrogen primed, immature, female rabbits An antigestagen should, by analogy with the definition of an antioestrogen, be able to inhibit the effect of administered progesterone upon the endometrium

A substance can be called luteotrophic if it increases the production of progesterone by the corpora lutea An antiluteotrophic substance should be able to inhibit the effect of injected LTH on the progesterone production of corpora lutea This definition, though ideal, is unfortunate in view of the confusion about what LTH really is (see page 14) It is therefore necessary at present to accept a more vague definition an antiluteotrophic substance should *in vivo* be able to reversibly reduce progesterone synthesis in corpora lutea or placentas Whilst both definitions include substances which reduce the corpora lutea cells' sensitivity to LTH, reduce the amount of progesterone precursors, or inhibit or block essential enzyme systems in the ovary, the latter definition also includes substances which reduce the synthesis or secretion of the hypothetical LTH or increase the rate of its breakdown

The antiovulatory properties of the oestrogens are assumed to be based upon reduced gonadotrophin secretion by the pituitary The antiovulatory effect demonstrated histologically in the toxicological experiments with high doses of F6103 and F6066 may represent a gonadotrophin inhibition due to the oestrogenic properties of these substances The effects of F6066 and F6103 in the experiments where seven injections were given weekly *may* therefore have been antiovulatory at the highest dose levels but, if ovulation occurred, the tubal transport mechanism, amongst other factors, could have been affected by the oestrogenic properties of the F substances DES was also effective in this test The result of the abortion test in rats on days 1–2, where dienoestrol and F6103 were very active, may also depend upon an effect on tubal transport

The effect of the F substances upon the rabbit uterus is antigestagenic, since F6066 and F6103 counteracted the effect of injected progesterone This antigestagenic effect failed in part when the F6066 dosage was held constant at 1 mg/kg whilst the dose of progesterone was doubled or quadrupled Conventional oestrogens were also antigestagenic in this test

be equally active on days 3-4 and days 11-12 in rats, in contrast to the results in mice When given to rats on days 17-18, the abortifacient effects of F6103 and ethynyl oestradiol were equally strong as calculated in mg/kg (table 9)

The ratios between the *antifertility effects*, both after seven times and twice weekly administration, and *oestrogenicity* also show that F6066 and F6103 have a stronger antifertility effect than DES in relation to oestrogenicity

Having established that the F substances possess some *abortifacient property* which is not included in their oestrogenic properties, it remains to inquire what this property is

Whole animal autoradiography with F6066 and F6060 revealed *the remarkable* observation that these substances became substantially and selectively (apart from the liver) localized in the corpora lutea of mice in late pregnancy and some, but not all, corpora lutea of the non pregnant females used This specific localization cannot be explained by the lipophilic properties of the substances, since there was no sign of any special localization in the body fat Historically speaking, the demonstration of localization in the corpora lutea was the main motivation for the later antifertility studies with F6066 and F6103

Among the large number of substances studied by whole body autoradiography at the Department of Pharmacology of the Royal Veterinary College in Stockholm such a distinct localization in the corpora lutea has only been observed with steroid hormone precursors (ULLBERG pers comm , APPELGREN 1967) Previous studies on oestrone, oestradiol and stilboestrol (ULLBERG & BENGTSSON 1963, BENGTSSON & ULLBERG 1963) have shown an accumulation of all three in the granulosa layer of large follicles, adrenal cortex, endometrium, interstitial tissue of the testes and ovaries and *in the corpora lutea of animals treated with stilboestrol but not in oestrone or oestradiol treated animals* Figure 5 of BENGTSSON & ULLBERG (1963) shows localization of stilboestrol in the corpora lutea the activity seems to be on the same level as in the kidney The corresponding autoradiograms obtained with F6066 (fig 4) show much greater activity in the corpora lutea than in the kidney

It thus seems reasonable to assume that F6066 interferes with progesterone synthesis The same may tentatively be said of F6103, although this substance has not been investigated autoradiographically The demonstration of localization in corpora lutea and antifertility activity led LARSSON & *STENSSON (1967)* to carry out *in vitro* experiments on the steroid biogenesis which leads to progesterone They *demonstrated* (page 11) inhibition of progesterone *synthesis* by both F6060 and F6204 (the dihydroxy substances were used on *account of* their solubility) which cannot be correlated with the *oestrogenicity of these* substances Oestradiol 17β had no inhibitory effect *under the same* experimental conditions, dihydroxydiethylstilboestrol had *the same inhibitory* effect as F6060 at the same molar concentration, but the *former* substance is about

Table 21

Ratios for antifertility effect/oestrogenic effect in rats and mice

	s c utero-trophic effect ED[1]) dose/day per animal γ	s c vagino-trophic effect ED50 dose/day per animal γ	ED50 for abortion mg/kg orally dosed days		Antifer-tility ED50 7 × week-ly mg/kg s c	Antifer-tility ED50 2 × week-ly mg/kg s c	Ratio 3–4/ oestro-genicity	Ratio 11–12/ oestro genicity	Ratio 11–12/3–4	Ratio 7 ×/ oestro-genicity	Ratio 2 ×/ oestro-genicity
			3–4	11–12							
Rats											
Ethynyloestradiol		0.1	0.2	0.6			2000	6000	3		
Dienoestrol		0.9	0.03	0.3			30	300	10		
DES		0.5	0.03	0.3			60	600	10		
F6066		800	20	50	0.001	0.008	30	60	3	2	20
F6103		20	2	4	0.5	2[2])	100	200	2	0.6	3
EB s c		0.2	0.006	0.006	⩽0.05	≤0.1	30	30	1	⩽3	≤5
Mice											
Ethynyloestradiol	0.04		0.01	>2			300	>40000	>160		
Dienoestrol	0.03		0.003	>0.2			100	> 5000	>60		
DES	0.02		0.01	0.2			500	10000	20		
EB s c	0.02		0.002	0.04			100	2000	20		
F6066	20		5	>80			300	>4000	>10		
F6103	0.4		0.3	2			1000	5000	7		

[1]) ED corresponds to the average uterine weight, which is reached after administration of 0.018 γ EB per animal

[2]) Estimated from Hanngren, Ullberg & Einer-Jensen (1965 b)

tributed to foetal excretion, even if the concentration in the foetus was inconsiderable The distribution picture of ^{14}C-F6060 and ^{14}C-F6066 in placenta and yolk sac is very similar to the distribution picture of Δ^5-3β hydroxysteroid dehydrogenase on fig 1 in DEANE *et al* (1962) An accumulation in the progesterone producing giant cells and interference with the progesterone production may therefore be possible, but a micro-autoradiographic study has not been performed

LARSSON (pers comn) has demonstrated an inhibition of progesterone production in the human placenta by F6060 in vitro

The foetus also plays a role in steroid biogenesis, especially in hydroxylation (DICZFALUSY 1965) However, only a very low concentration of the F substances can be demonstrated in the foetuses autoradiographically, so that an effect on steroid hydroxylation is improbable, especially since LARSSON (personal communication) has demonstrated no effect *in vitro* of the F substances on hydroxylations

As earlier mentioned F6066 and F6103 affect steroid dehydrogenases, but do not seem to influence hydroxylation processes An inhibition of 11β-hydroxylation in the adrenal cortex can be seen after administration of Metopiron (SU 4885) The synthesis of certain corticoids, especially cortisol, corticosterone and aldosterone is thereby hindered Metopiron is used as a test substance in the assessment of the pituitary's capacity to increase ACTH secretion (NEHER & KAHNT 1965)

It is advantageous if a drug which affects steroid metabolism exerts as specific an effect as possible, preferably influencing *one* late step in synthesis. F6066, F6103 and Metopiron seem to fulfil this requirement, which should theoretically imply a lower frequency of undesirable side effects

Triparanol (Mer 29), which inhibits the hydroxylation of 24-dehydrocholesterol (desmosterol) to cholesterol, was used to reduce blood cholesterol levels by continuous administration and according to certain theories, reduce the risk for arteriosclerosis (OAKS, LISAN & MOYER 1959) The substance affects such an early stage of synthesis that the adrenal production of steroids is also reduced an inhibition which can only partly be compensated by increased secretion of ACTH (GORDON, MAUER, CEKLENIAK & PARTRIDGE 1963) Clinical application of this substance has been abandoned due to the side effects

Three months of treatment with F6066 (EINER JENSEN 1965) or F6103 (unpublished) increases the weight of the adrenals of male rats by 30–50% In the autoradiographic work with F6066 (page 74) a very strong localization in the corpora lutea was observed, together with a weaker localization in the adrenal cortex This is a favourable distribution because LARSSON & STENSSON (1967) showed an inhibitory effect on the conversion rate of pregnenolone in the rat adrenals in *in vitro* trials Too strong localization in the adrenals could jeo-

1000 times more oestrogenic than the latter It is striking that stilboestrol shows some localization in corpora lutea and some inhibition of progesterone production, whilst oestradiol exhibits *neither* of these attributes Whether or not the *in vivo* concentration of F6066 (F6060) or F6103 (F6204) in the corpora lutea is sufficient to inhibit progesterone production is unknown, since a quantitative evaluation of the concentrations achieved in the autoradiographic studies was not possible APPELGREN (1967) has histochemically on whole body sections demonstrated a very selective activity in the corpora lutea of the enzyme which transforms pregnenolone into progesterone, Δ^5-3β-hydroxysteroid dehydrogenase He also showed that this enzyme activity was inhibited by F6103

On this background it is highly probable that a major factor involved in the antifertility effect of the F substances is an inhibition of progesterone production Such an effect was earlier described for ergocornine, but this was not possible to verify (page 19), and has now also been described for stilboestrol, but the author is not aware of any other publications concerning non-steroidal substances with this property Nevertheless since stilboestrol can inhibit progesterone synthesis *in vitro* and since LARSSON (personal communication) has demonstrated a similar effect with other diphenylethenes, it is probable that other chemically related non-steroid oestrogens also possess this property Inhibition of 3β-hydroxysteroid dehydrogenase can be obtained with steroids (e g FERRARI & ARNOLD 1963, GOLDMAN 1967)

The results of abortion tests in rats and mice with simultaneous administration of F6066 and progesterone (page 43) suggest that inhibition of progesterone synthesis is only partially responsible for the antifertility properties of the F substances If abortion, especially on days 11–12 and later, is due principally to an inhibition of progesterone synthesis, injections of progesterone, but not of HCG, prolactin or pregnenolone, should theoretically maintain pregnancy In our experiments, however, progesterone did not prevent abortion The failure to achieve an effect may possibly be a parallel to the disputable effect of progesterone administration to women in threatened abortion Our experience corresponds to that of MARTIN (1963) with oestradiol + progesterone, although he only investigated the period before implantation

During the first part of pregnancy, the corpora lutea are the main source of the progesterone which is essential for the maintenance of pregnancy In primates, progesterone production is taken over by the placenta to such an extent that ovariectomy can be performed without the risk of abortion Placental production of progesterone also appears to occur in the mouse, although luteal production does not become superfluous (see page 15)

The strong localization of F6066 (or radioactive metabolites) in the yolk sac epithelium, demonstrated in the autoradiograms, increased with time, and after four hours was the highest in the whole body Possibly this can be at

tributed to foetal excretion, even ... concentration in the foetus was in-
considerable The distribution pictu...
and yolk sac is very similar to the ... (1962) An accumulation in the pro-
dehydrogenase on fig 1 in DEANE *et al* (1962) An accumulation in the pro-
gesterone producing giant cells and interference with the progesterone pro-
duction may therefore be possible, but a micro-autoradiographic study has not
been performed

LARSSON (pers comn) has demonstrated an inhibition of progesterone pro-
duction in the human placenta by F6060 in vitro

The fo ... steroid biogenesis especially in hydroxylation
(DICZFA ...
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munication) has demonstrated no effect *in vitro* of the F substances ...
lations

As earlier mentioned F6066 and F6103 affect steroid dehydrogenases, but do not seem to influence hydroxylation processes An inhibition of 11β-hydroxylation in the adrenal cortex can be seen after administration of Metopiron (SU 4885) The synthesis of certain corticoids, especially cortisol, corticosterone and aldosterone is thereby hindered Metopiron is used as a test substance in the assessment of the pituitary s capacity to increase ACTH secretion (NEHER & KAHNT 1965)

It is advantageous if a drug which affects steroid metabolism exerts as specific an effect as possible, preferably influencing *one* late step in synthesis. F6066, F6103 and Metopiron seem to fulfil this requirement, which should theoretically imply a lower frequency of undesirable side effects

Triparanol (Mer 29), which inhibits the hydroxylation of 24-dehydrocholesterol (desmosterol) to cholesterol, was used to reduce blood cholesterol levels by continuous administration and according to certain theories, reduce the risk for arteriosclerosis (OAKS, LISAN & MOYER 1959) The substance affects such an early stage of synthesis that the adrenal production of steroids is also reduced, an inhibition which can only partly be compensated by increased secretion of ACTH (GORDON, MALER, CEKLENIAK & PARTRIDGE 1963) Clinical application of this substance has been abandoned due to the side effects

Three months of treatment with F6066 (EINER JENSEN 1965) or F6103 (unpublished) increases the weight of the adrenals of male rats by 30-50% In the autoradiographic work with F6066 (page 74) a very strong localization in the corpora lutea was observed, together with a weaker localization in the adrenal cortex This is a favourable distribution because LARSSON & STENSSON (1967) showed an inhibitory effect on the conversion rate of pregnenolone in the rat adrenals in *in vitro* trials Too strong localization in the adrenals could jeo-

1000 times more oestrogenic than the latter It is striking that stilboestrol shows some localization in corpora lutea and some inhibition of progesterone production, whilst oestradiol exhibits *neither* of these attributes Whether or not the *in vivo* concentration of F6066 (F6060) or F6103 (F6204) in the corpora lutea is sufficient to inhibit progesterone production is unknown, since a quantitative evaluation of the concentrations achieved in the autoradiographic studies was not possible APPELGREN (1967) has histochemically on whole body sections demonstrated a very selective activity in the corpora lutea of the enzyme which transforms pregnenolone into progesterone, Δ^5-3β-hydroxysteroid dehydrogenase He also showed that this enzyme activity was inhibited by F6103

On this background it is highly probable that a major factor involved in the antifertility effect of the F substances is an inhibition of progesterone production Such an effect was earlier described for ergocornine, but this was not possible to verify (page 19), and has now also been described for stilboestrol, but the author is not aware of any other publications concerning non steroidal substances with this property Nevertheless since stilboestrol can inhibit progesterone synthesis *in vitro* and since LARSSON (personal communication) has demonstrated a similar effect with other diphenylethenes, it is probable that other chemically related non-steroid oestrogens also possess this property Inhibition of 3β-hydroxysteroid dehydrogenase can be obtained with steroids (e g FERRARI & ARNOLD 1963, GOLDMAN 1967)

The results of abortion tests in rats and mice with simultaneous administration of F6066 and progesterone (page 43) suggest that inhibition of progesterone synthesis is only partially responsible for the antifertility properties of the F substances If abortion, especially on days 11–12 and later, is due principally to an inhibition of progesterone synthesis, injections of progesterone, but not of HCG, prolactin or pregnenolone, should theoretically maintain pregnancy In our experiments, however, progesterone did not prevent abortion The failure to achieve an effect may possibly be a parallel to the disputable effect of progesterone administration to women in threatened abortion Our experience corresponds to that of MARTIN (1963) with oestradiol + progesterone, although he only investigated the period before implantation

During the first part of pregnancy, the corpora lutea are the main source of the progesterone which is essential for the maintenance of pregnancy In primates, progesterone production is taken over by the placenta to such an extent that ovariectomy can be performed without the risk of abortion Placental production of progesterone also appears to occur in the mouse, although luteal production does not become superfluous (see page 15)

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in patients treated for prostate cancer with Estradurin® (an oestradiol depôt preparation), DES and F6066 After treatment with the recommended dose of Estradurin, only a few changes were seen in testicular histology With DES, a pronounced shift to the left of spermiogenesis was obtained and it was difficult to demonstrate Leydig cells After F6066 treatment there was pronounced hypertrophy and hyperplasia of the Leydig cells, which appeared large and rich in lipids, and spermiogenesis showed a shift to the left. We have histologically examined testicles from rats, rabbits and dogs treated continuously for 2–3 months with F6066 of F6103 (e g in toxicological experiments) but have not observed an equivalent Leydig cell hyperplasia, although we have seen a hyperplasia of the interstitial tissue of the ovaries (unpublished) Such a hyperplasia, as well as Leydig cell hyperplasia, can occur together with a reduction in organ weight, since this is dominated by weight changes in the spermiogenetic tissue and corpus luteum tissue This group of findings supports the hypothesis that the F substances, to a greater extent than DES, exert their effect by interfering with steroid metabolism at an ovarian or testicular level, whilst their influence on the pituitary is presumably less important

A substance which can interrupt early pregnancy in women, surely and without complications, is desired by many as an aid in family planning It is established in the literature that large doses of oestrogen do not induce abortion in women once the placenta is developed SMITH & SMITH (1949) have shown that when large doses of stilboestrol are given (from 12 5 mg daily in week 12 up to 125 mg daily in the last month of pregnancy), the frequency of side effects and abortions is reduced, if there is any effect on pregnancy at all However, F6103 theoretically fulfils many of the criteria required of a pregnancy interrupting drug The hypothesis has been tested on pregnant women by ENGSTROM, as mentioned on page 12

It is not possible to compare F6103's capacity for interrupting established pregnancy in women with that of other substances, since no such studies exist in the literature

F6103 may not appear to be of practical use for the induction of abortion in women with a diagnosed pregnancy pregnancy may not continue but may in some cases be converted to missed abortion It is possible that this undesirable state could be avoided by subsequent treatment with oestrogen + gestagen, possibly followed by oxytocin A different period of treatment, which is theoretically preferable, is during the days immediately preceding expected menstruation

In summary it may be said that the weak, non steroidal oestrogens F6066 and F6103 are, amongst other things, abortifacient in animal experiments Some of their antifertility effects *are* based upon the weak oestrogenic properties exhibited by both substances. This is *relevant in* the tubal transport period and to the anti Clauberg effect, and is *partially* responsible for the anti-

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pardize corticoid synthesis, for which progesterone is a precursor No sign of an influence on 17-ketosteroids or 17-ketogenic steroids has been observed during use of F6066 in human clinical trials (PERSSON 1965a) Thus if an inhibition occurs in the dosages used, it is compensated without difficulty by the pituitary and adrenals

No teratogenic effect has been demonstrated for F6066 in mice, rats or humans, nor for F6103 in mice, rats, rabbits or dogs The weak penetration of the placenta by ^{14}C-F6066 suggests that there is a relatively minor risk for direct action upon the foetus The lack of teratogenicity is an important condition for pregnancy interrupting experiments in humans

The pharmacological analysis of the inhibition of progesterone production is only partially completed, since we have not had access to an experimental model where the influence on luteal production can be analysed on a target organ other than the uterus In the pregnant animal we can record the sum of the effects on the uterus and corpora lutea, and in the ovariectomized or im mature animal we can record the direct effects on the uterus The mammary gland may possibly be an organ whose reactions can be studied in parallel with the uterus

There are distinct differences between the effects of conventional oestrogens and the F substances in animal experiments, even outside the antifertility field EINER-JENSEN (1965) reported that a 40% weight increase of musculus levator ani in castrated male rats followed treatment with DES or EB, whilst the oestrogen-equivalent doses of F6066 and F6060 did not alter the weight of this muscle

If DES or EB are administered continuously to castrated or intact, male or female animals, the pituitary weight increases as compared with that in control animals After subcutaneous administration of 20 µg/kg DES for four weeks to 20 growing male rats, the mean pituitary weight was 7 1 mg, after 2 mg/kg F6103 it was 4 8 mg The mean pituitary weight of control animals was 6 2 mg Since the mean body weight at sacrifice was 148, 127 and 173 grams for the three groups respectively, the ratios between pituitary weight and body weight were 47, 38 and 36 respectively In the corresponding females treated with 40 µg/kg DES, 4 mg/kg F6103 or 40 µg/kg DES + 4 mg/kg F6103, oil treated control animals and oil treated oophorectomized animals, the ratios between pituitary weight and body weight after four weeks of treatment were 67, 43, 48 46 and 47 respectively In both female and male animals, the difference in pituitary weight between F6103 and DES treated rats is statistically significant ($P < 0\ 05$) Despite the differences in weight of the pituitaries, the testicles and ventral prostate of the male or ovaries and uterus of the female decreased more in weight in F6103 treated than in DES treated animals (unpublished investigations from our laboratories)

NYLANDER, PERSSON & TERNER (1967) have studied testicular morphology

Summary

The present investigation concerns the antifertility properties of Bis-(p-acetoxyphenyl)cyclohexylidene methane, F6066, and Bis-(p-acetoxyphenyl)-2-methyl-cyclohecylidene methane, F6103

When daily, subcutaneous injections of F6066, F6103 or diethylstilboestrol in oil were given to female rats for one week before and three weeks during contact with males, there was a dose dependent reduction in the number of completed pregnancies and in the number of living offspring. The antifertility effect was partly reversible, since within two weeks after the final injection some of the females became pregnant F6103 was more than ten times as active as F6066

In a subsequent experiment with F6103 and DES, the number of injections was reduced to two per week, given on consecutive days The first injection was given three days after housing of the females with males, and the injections were repeated on the same days of the week for three weeks, the last injection being given three days after the male was removed In this experiment too, there was a dose-dependent reduction in the number of completed pregnancies and of young Vaginal smears were examined daily, some of those females which did not give birth to young had mated before the first injection was given When the males were removed there was a marked increase in the frequency of oestrous This increase was still demonstrable at the termination of the experiment, three weeks after removal of the males Blood was observed in vaginal smears from pregnant animals in all groups on one or several days between the 12th and 16th days of gestation The antifertility effect of the F substances was stronger than that of DES relative to their vaginotrophic effect both after daily and twice weekly administration

F6066 and F6103 hindered completion of a normal pregnancy when given to pregnant rats and mice The same effect was shown with F6103 in rabbits and bitches

F6103 was an effective abortifacient when a few mg/kg were given during various two-day periods from day 1 to day 12 of rat or mouse pregnancy Ethynyl oestradiol, DES and dienoestrol were also effective but, compared with F6103 relatively less active on days 11–12 than on days 3–4, although more active than F6103 in both periods reckoned in mg/kg. Administration of progesterone, pregnenolone, and preparations containing prolactin or HCG did not

fertility action of the substances in early pregnancy Their abortifacient effects in the later period of pregnancy may be partially ascribed to a property not shared by the steroid oestrogens This property can be related to the localization of the substances in active corpora lutea and the inhibition of progesterone production demonstrated by LARSSON & STENSSON (1967) and APPELGREN (1967) The relative importance of the two abortifacient properties depends upon in which stage of pregnancy abortion is induced and in which species

Conclusion

F6066 and F6103 have shown antifertility properties when given subcutaneously twice or seven times weekly to female rats housed with males

It has been shown that F6066 and F6103 both have an abortifacient effect when given to rats and mice for two days during pregnancy F6103 is relatively more active on days 11 and 12 of pregnancy than on days 3 and 4, compared with diethylstilboestrol, dienoestrol, ethynyl oestradiol and oestradiol benzoate F6103 has also been tested on days 17 and 18, when it proved equally active as ethynyl oestradiol in mg/kg

F6103 is abortifacient in rabbits throughout pregnancy and in dogs up to at least the 10th day after mating F6066 and F6103 antagonize the effect of administered progesterone in the Clauberg test in female rabbits F6066 becomes localized very specifically in the hormonally active corpus luteum

F6066 in subabortive doses has shown no sign of teratogenic properties in pregnant mice and rats neither has F6103 in pregnant mice, rats, rabbits and bitches

Interpretation of the above results indicates that both F6066 and F6103 are antifertile substances when given after ovulation The substances may affect all phases of pregnancy egg transport, decidua formation, implantation and even later phases when the foetus has established placental connections with the mother In early pregnancy, the effect suggests that of the other oestrogens mentioned above, but F6103 becomes relatively more effective as pregnancy progresses On the basis of the localization of F6066 in active corpora lutea, the hypothesis is put forward that this is because of interference with progesterone synthesis and that F6066 and F6103, as well as being weak, non steroidal oestrogens, can be regarded as antiluteotrophic substances

prevent abortion in the doses used F6103 was as active as ethynyl oestradiol, reckoned in mg/kg, when given on days 17 and 18 to rats

In teratological experiments with F6066 and F6103 in mice and rats, the highest doses used induced abortion in accordance with the results of the above experiments Subabortive doses produced no demonstrable foetal malformations Young from rabbits and bitches treated with F6103 during pregnancy showed no signs of malformation

F6066 and F6103, given subcutaneously, antagonized the effect of simultaneously injected progesterone on the uterus of immature rabbits (anti Clauberg test) DES and oestradiol benzoate had the same effect

The distribution of ^{14}C labelled F6066 and its dihydroxy equivalent, F6060, was studied by whole body autoradiography in the mouse The most striking finding was a rapid and selective accumulation in the corpora lutea A selective and progressively increasing accumulation was also seen in the visceral yolk sac epithelium A slight accumulation was found in the hypophysis, the adrenal cortex, the endometrium, the interstitial tissue of the testis and ovary, the epididymis and the mucosa of the seminal vesicles No accumulation was found in the prostate There was a very low concentration in the foetus during the whole course of observation In addition to the radioactivity in the liver, bile and intestinal lumen, the only organs showing any retention 24 hours after administration were the corpora lutea and yolk sac epithelium

References

Adlercreutz, H , C J Johansson & T Luukkainen Gas chromatographic and mass spectrometric studies in the influence of metabolites of bis(p acetoxyphenyl)cyclohexylidenemethane on the estimation of estrogens in urine *Ann Med exp Fenn* 1967, 45, 269–276

Alden, R H Aspects of the egg – ovary – oviduct relationship in the albino rat *J Exp Zool* 1942, 90, 159–169

Amoroso, E C & C A Finn In The ovary Ed by S Zuckerman Vol 1 N Y 1962a, pp 477–480

Amoroso, E. C & C A Finn In The ovary Ed by S Zuckerman Vol 1 N Y 1962b, pp 483–488

Appelgren, L E Sites of steroid hormone formation Autoradiographic studies using labelled precursors *Acta physiol scand* 1967, suppl 301

Arnold, M , M Berger, M Keller, R H H Richter & A Vettwiller Clinical and biochemical studies in patients treated with bis (p acetoxyphenyl)cyclohexylidenemethane (Compound F6066) *Acta endocr* (Kbh) 1967, suppl 119, Abstr 207

Astwood E. B & R O Greep A corpus luteum stimulating substance in the rat placenta *Proc Soc exp Biol Med* 1938, 38, 713–716

Banik, U K. & G Pincus Estrogens and transport of ova in the rat. Proc. Soc. exp Biol Med 1964, 4, 1032–1034

Beaudoin A R & J Roberts Serum proteins and teratogenesis *Life Sci* 1965, 4, 1353–1358

Beck, F & J B Lloyd Dosage response curves for the teratogenic activity of trypan blue *Nature* 1964, 201, 1136–1137

Bencze, W L R W J Carney, L I Barsky, A A Renzi, L Dorfman & G de Steven Synthetic estrogens and implantation inhibitors. *Experientia* 1965, 21, 261

Ben David, M & F G Sulman The mechanism of systemic deciduoma production *Arch int Pharmacodyn* 1966, 159 161–169

Bengtsson G & S Ullberg The autoradiographic distribution pattern after administration of diethylstilboestrol compared with that of natural oestrogens *Acta endocr* (Kbh) 1963 43, 561–570

Bennet J P , K E. Kendle D K Vallance & B H Vickery A comparison of the antifertility and hormonal activities of some new synthetic oestrogens *Acta endocr* (Kbh) 1966 53 443–454

Bjoro K Ovarialfunksjonens reguleringsmekanismer *T norske Lægeforen* 1966, 86, 1704–1710

Bonnevier P , M Fog & P Rus Om svangerskabsforebyggelse *Bibl Læger* 1966, 158 5, 485–515

Borell, U & G Rybo In Om hormoner och hormonterapi *Gbg* 1962, pp 82–83

Brock N & Th von Kreybig Teratogenese als pharmakologisch toxikologisches Problem *Arzneimittel Forsch* 1964, 14, 655–664

Chang, M C Effects of oral administration of medroxyprogesterone acetate and ethynyl

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NIELS EINER-JENSEN

Finn, C. A Oestrogens and the decidual cell reaction of implantation in mice *J Endocr* 1965, 32, 223–229

Flerkó, B Zur Hypothalamischen Steuerungen der gonadotrophen Funktion der Hypophyse. *Acta morph Acad Sci hung* 1954, 4, 475–492.

Flerkó, B & J Szentagothai Oestrogen sensitive nervous structures in the hypothalamus *Acta endocr* (Kbh) 1957, 26, 121–127

Gillman, T & F Beck In A symposium on embryopathic activity of drugs Ed J M Robson, F Sullivan and R L Smith, London 1965, 35–37

Ginther, O J Local utero ovarian relationships. *J Animal Sci* 1967, 26, 578–585

Goldman, A S Inhibition of 3β hydroxysteroid dehydrogenase from pseudomonas testosteroni by various estrogenic and progestinic steroids. *J Clin Endocr* 1967, 27, 320–324

Gordon, S , S Mauer, W F Ceklemak & R Partridge Mechanism of triparanol induced adrenal hypertrophy and reduced adrenal function *Endocrinology* 1963, 72, 643–648

Gregg, N M Congenital cataract following German measles in the mother *Trans ophthal Soc Aust* 1941, 3, 35 46

Grover, P K , H P C Chawla, N Anand, V P Kamboj & A B Kar New antifertility agents. 2,3 diphenylbenzofurans *J med Chem* 1965, 8, 720–721

Hamburger, C In Hormone assay, Ed by C W Emmens, N Y 1950, p 175

Hanngren, Å , N Einer Jensen & S Ullberg Distribution pattern of a non steroid compound with endocrine actions, ^{14}C bis(p hydroxyphenyl)cyclohexylidenemethane (Compound F6060) and its diacetate (Compound F6066) *Acta endocr* (Kbh) 1965a, 50, 35–43

Hanngren, Å , N Einer Jensen & S Ullberg Specific uptake in corpora lutea of a non-steroid substance with anti gestagenic properties *Nature* 1965b, 208, 461–462

Harper, M J K & A L Walpole Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. *Nature* 1966, 212, 87

Harper, M J K & A L Walpole A new derivate of triphenylethylene Effect on implantation and mode of action in rats *J Reprod Fertil* 1967, 13, 101–119

Harris, G W , M Reed & C P Fawcett Hypothalamic releasing factors and the control of pituitary function *Brit med Bull* 1966, 22, 266–272.

Holtkamp, D E , J G Greslin, C A Root & L J Lerner Gonadotrophin inhibiting and anti fecundity effects of chloramiphene *Proc Soc exp Biol* 1960, 105, 197–201

Ishihama, A *Yokohama med Jour* 1959, 10, 89–101 Ref *Clinic Obstet Gynec* 1964, 7, 814

Johnsen, S G De antikonceptionelle pillers hormonala egenskaber *Ugeskr Læg* 1966, 128 1257–1260

Johnson L , G Nylander & S Wikström Effect of a new diphenylethylene derivate on prostatic function in dogs *Acta Soc Med Upsalien* 1966, 71, 311–333

Karnaky, K J Dosage of stilbestrol (letter to the editor) *Amer J Obstet Gynec* 1950, 60, 1182–1184

Kincl, F A & R. I Dorfman Influence of progestional agents on the genetic female foetus of orally treated pregnant rats. *Acta Endocr* (Kbh) 1962, 41, 274–279

Källén B *Läkemedelsframkallad teratogenes Opusc med.* 1967, 12 suppl. 3

Larsson, H The metabolic fate of bis(p-hydroxyphenyl)cyclohexylidenemethane – F6060 – and its diacetate – F6066. *Acta pharmacol* (Kbh) 1965, 23, 337–359

Larsson H & M Stensson Effect of bis(p-hydroxyphenyl)-cyclohexylidenemethane (F6060) on the conversion of pregnenolone to Δ^4 3 ketosteroids in vitro *Acta endocr* (Kbh) 1967, 55, 673–684

Lednicer D , J C Babcock, S C Lyster, J C Stucki & G W Duncan Derivatives of 2,3-diphenylindenes as antifertility agents. *Chem Ind* 1961, 2098 2099

oestradiol on the transportation and development of rabbit eggs *Endocrinology* 1966, 79, 939–948

Clauberg, C Zur Physiologie und Pathologie der Sexualhormone, im besonderen des Hormons des Corpus Luteum *Zbl Gynak 1930, 54, 2757–2770*

Cook, J W, E C Dodds, C L Hewett & W *Lawson* The oestrogenic activity of some condensed ring compounds in relation to their other biological activities *Proc roy Soc B* 1934, 114, 272–286

Corner, G W The hormones in human reproduction Princeton 1946, pp 103–134

Cousins, F B & C P M Geary A sex determined renal calcification in rats *Nature* 1966, 211, 980–981

Deane, H W, B L Rubin, E C Driks, B L Lobel & G Leipsner Trophoblastic giant *cells* in placentas of rats and mice and their probable role in steroid hormone production *Endocrinology* 1962, 70, 407–419

Diczfalusy, E Månniskofostrets roll vid graviditetens endokrina reglering, in 20 års medicinsk forskning Sthm 1965, pp 362–373

Diczfalusy, E Verkningsmekanismer vid hormonell kontraception *Lak Tidn* 1967 64, 4809–4839

Dodds, E C, L Golberg, W Lawson & R Robinson Oestrogenic activity of certain synthetic compounds *Nature* 1938, 141, 247–248

Dodds, E C, L Golberg, W Lawson & R Robinson Synthetic oestrogenic compounds related to stilbene and diphenylethane Part I *Proc roy Soc B* 1939, 127, 140–166

Dodds, E C, L Golberg E I Grunfeld, W Lawson, C M Saffer & R Robinson Synthetic oestrogenic compounds related to stilbene and diphenylethane Part II *Proc roy Soc B* 1944–45, 132, 83–101

Dodds, E C & W Lawson Molecular structure in relation to oestrogenic activity Compounds without a phenanthrene nucleus *Proc roy Soc B* 1938, 125, 222–232

Duncan, G W, J C Stucki, S C Lyster & D Lednicer An orally effective mammalian antifertility agent *Proc Soc exp Biol Med* 1962, 109, 163–166

Eckstein, P In The ovary Ed by S Zuckerman Vol 1 N Y 1962, p 319

Eckstein, P Intrauterine contraceptive devices The scientific background *Proc roy Soc Med* 1967, 60, 12–15

Einer Jensen, N Bis(p acetoxyphenyl)cyclohexylidenemethane (F6066) a nonsteroidal compound with pronounced effect on the reproductive system *Acta pharmacol* (Kbh) 1965, 23 365–382

Einer Jensen, N The planning of a new industrial pharmacological laboratory and the activity in this laboratory during six months Abstract from the XXth Scandinavian Pharmacological Meeting in Turku 1966

Einer Jensen, N Effects on pregnancy and offspring in rats and mice of bis(p acetoxyphenyl)cyclohexylidenemethane (F6066) *Acta pharmacol* (Kbh) 1967a, 25, 65–79

Einer Jensen, N Induction of ovulation by bis(p acetoxyphenyl)cyclohexylidenemethane (F6066) in the dog *Nord Vet-Med* 1967b, 19, 236–239

Eisenfeld, A & J Axelrod Selectivity of estrogen distribution in tissues *J Pharmacol exp Ther* 1965, 150, 469–475

Emmens C W Endocrine factors in implantation in changing patterns in fertility Conference of the international planned parenthood federation 7 Proceedings Amsterd 1964, pp 553–557

Falck, B Site of production of oestrogen in rat ovary as studied in micro transplants *Acta physiol scand* 1959, 47, suppl 163

Ferrari, R A & A Arnold Inhibition of β hydroxysteroid dehydrogenase I Structural characteristics of some steroidal inhibitors *Biochim Biophys Acta* 1963, 77, 349–364

Persson B H Clinical effect of bis(p acetoxyphenyl)cyclohexylidenemethane (Compound F6066) on menstrual disorders *Acta Soc Med Upsalien* 1965b, 70, 71–81
Pincus, G The control of fertility N Y 1965, pp 217–235
Rothchild, I Interrelations between progesterone and the ovary, pituitary and central nervous system in the control of ovulation and the regulation of progesterone secretion *Vitam and Horm* 1965, 23, 209–305
Roux C & R Dupuis Action tératogène du triparanol *C R Soc Biol* 1961, 155, 2255–2257
Rudel, H W & F A Kincl The biology of anti fertility steroids *Acta endocr* 1966, 51 suppl 105
Ryan, K J & R V Short Formation of estradiol by granulosa and theca cells of the equine ovarian follicle *Endocrinology* 1965, 76, 108–114
Schoug, J S & R K Meyer Effect of antiestrogens on estrogen induced ova implantation in the ovariectomized rat *Fertil and Steril* 1965, 16, 106–112
Segaloff, A In Methods in hormone research Ed R I Dorfman Vol 2 N Y 1962, pp 609–611
Shelesnyak, M C Nidation of the fertilized ovum *Endeavour* 1960, 19, 81–86
Shelesnyak, M C & P F Kraicer A physiological method for inducing experimental decidualization of the rat uterus Standardization and evaluation *J Reprod Fertil* 1961, 2, 438–446
Shelesnyak, M C, B Lunenfeld & B Hornig Studies on the mechanism of ergocornine interference with decidualization and nidation III Urinary steroids after administration of ergocornine to women *Life Sci* 1963, 1, 73–79
Smith, O W & G V S Smith The influence of diethylstilbestrol on the progress and outcome of pregnancy as based on a comparison of treated with untreated primigravidas *Amer J Obstet Gynec* 1949, 58, 994–1009
Solomon J B Relative growth of trophoblast and tumour cells co implanted into isogenic mouse testes and the inhibitory action of 'Methohexate *Nature* 1966, 210, 716–718
Southam A L Historical review of intra uterine devices In Intra uterine contraception 2nd international conference, October 2–3, 1964, New York *Proceedings Amsterd* 1965 pp 3–5
Staples R E Effect of clomiphene on blastocyst nidation in the rat. *Endocrinology* 1966, 78 82 86
Terner N Ett nytt diphenylethylenderivat (F6066) Behandlingsresultat vid cancer pro statae *Nord Med* 1967, 77, 65
Ullberg S Autoradiographic studies on the distribution of labelled drugs in the body In International conference on the peaceful use of atomic energy 2 (Geneva 1958), Proceeding 24 248–254
Ullberg S & G Bengtsson Autoradiographic distribution studies with natural oestrogens *Acta endocr* (Kbh) 1963, 43, 75–86
Warnick A S J Gibson, M Vinegra & S Tolksdorf Ethynyl estradiol A potent orally active contraceptive in rats *Proc Soc exp Biol* 1964 116, 343–347
Velardo J T & F L Hisaw Quantitative inhibition of progesterone by estrogens in development of deciduomata *Endocrinology* 1951, 49 530–537
Wexler B C Reproductive defects and abnormal progeny of rats treated with triparanol Prevention of abnormalities by ACTH *Endocrinology* 1964, 74 64–78
Willier B H In Analysis of development Ed B H Willier *Philad* 1955, p 594
Wolff E In The ovary Ed S Zuckerman, Vol 2 N Y 1962, pp 81–129

Lednicer, D, S C Lyster & G W Duncan Mammalian antifertility agents Part II Basic ethers of 3,4 diphenylcoumarins *J med Chem* 1965, 8, 725–726

Lindner, H R, B Lunenfeld & M C Shelesnyak Plasma levels of progesterone and cortisol, and urinary pregnanediol excretion, during the post ovulatory phase of the cycle in normal and ergocornine treated women *Acta endocr* (Kbh) 1967, 56, 35–42

Lindner, H R & M C Shelesnyak Effect of ergocornine on ovarian synthesis of pro gesterone and 20 α hydroxy pregn 4 en 3 one in the pseudopregnant rat *Acta endocr* (Kbh) 1967, 56, 27–34

Lobel, B L, E Levy & M C Shelesnyak Dynamics of cellular interactions during progestation and implantation in the rat I Progestation, II Nidus formation, III Implantation *Acta endocr* (Kbh) 1967, suppl 123

Lorke, D Zur Metodik der Untersuchungen embryotoxischer und teratogener Wirkungen an der Ratte *Naunyn Schmiederberg s Arch exp Path Pharmack* 1963, 246, 147–151

Martin, L Interactions between oestradiol and progestogens in the uterus of the mouse *J Endocr* 1963, 26, 31–39

Mayer, G Recent studies on hormonal control of delayed implantation and super implantation in the rat *Mem Soc Endocr* 1959, 6, 76–83

McLaren, A & D Michie Congenital runts In A ciba foundation symposium on congenital malformations Ed G E W Wolstenholme & C M O Connor 1960, 178–198

McPhail, M K The assay of progestin *J Physiol* (Lond) 1934, 83, 145–156

Miquel, J F 1,1 di p hydroxyphenyl 2,2 diethylethylene, a new isomer of stilbestrol *Acta chem scand* 1958, 12, 274–280

Miquel, J F, H Wählstam, K Olsson & B Sundbeck Synthesis of unsymmetrical diphenylalkenes *J med Chem* 1963, 6, 774–780

Morris, J McL & G van Wagenen Compounds interfering with ovum implantation and development *Amer J Obstet Gynec* 1966, 96, 804–813

Morris, J McL, G van Wagenen, G D Hurteau, D W Johnston & R A Carlsen Compounds interfering with ovum implantation and development I Alkaloids and antimetabolites *Fertil and Steril* 1967, 18, 7–17

Morris, J McL, G van Wagenen, T McCann & D Jacob Compounds interfering with ovum implantation and development II Synthetic estrogens and antiestrogens *Fertil and Steril* 1967, 18, 18–34

Neher, R & F W Kahnt Modifiers of adrenocortical function International pharmaco logical meeting, Prague, August 1963 2 Complete official proceedings Symposium 4 Drugs and enzymes Ed B Brodie Prague 1965, pp 209–223

Nielsen, E H Veterinær embryologi Kbh 1965, pp 114–121

Nishihara, G Influence of female sex hormones in experimental teratogenesis *Proc Soc exp Biol Med* 1958, 97, 809–812

Nylander, G, B H Persson & N Terner Testis' morfologi vid östrogen terapi *Nord Med* 1967, 78, 1493–1494

Nylander, G & N Terner A new pituitary inhibiting compound in the treatment of prostatic cancer *Urol int* 1964, 17, 129–145

Oaks, W, P Lisan & J M Moyer Inhibition of cholesterol synthesis with the use of Mer 29 *Arch intern Med* 1959, 104, 527–531

Oppenheimer, W Prevention of pregnancy by the Graefenberg ring method *Amer J Obstet Gynec* 1959, 78, 446–454

Persson, B H The effect of bis(p acetoxyphenyl)cyclohexylidenemethane (Compound F6066) on hormone excretion in postmenopausal women *Acta Soc Med Upsalien* 1965a, 70, 1–16

Persson, B H Clinical effect of bis(p-acetoxyphenyl)cyclohexylidenemethane (Compound F6066) on menstrual disorders *Acta Soc Med Upsalien* 1965b, 70, 71–81

Pincus, G The control of fertility N Y 1965, pp 217–235

Rothchild, I Interrelations between progesterone and the ovary, pituitary and central nervous system in the control of ovulation and the regulation of progesterone secretion *Vitam and Horm* 1965, 23, 209–305

Roux, C & R Dupuis Action tératogéne du triparanol *C R Soc Biol* 1961, 155, 2255–2257

Rudel, H W & F A Kincl The biology of anti fertility steroids. *Acta endocr* 1966, 51 suppl 105

Ryan, K J & R V Short Formation of estradiol by granulosa and theca cells of the equine ovarian follicle *Endocrinology* 1965, 76, 108–114

Schloug, J S & R K Meyer Effect of antiestrogens on estrogen induced ova implantation in the ovariectomized rat *Fertil and Steril* 1965, 16, 106–112

Segaloff, A In Methods in hormone research Ed R I Dorfman Vol 2 N Y 1962, pp 609–611

Shelesnyak, M C Nidation of the fertilized ovum *Endeavour* 1960, 19, 81–86

Shelesnyak, M C & P F Kraicer A physiological method for inducing experimental decidualization of the rat uterus Standardization and evaluation *J Reprod Fertil* 1961, 2, 438–446

Shelesnyak, M C, B Lunenfeld & B Hornig Studies on the mechanism of ergocornine interference with decidualization and nidation III Urinary steroids after administration of ergocornine to women *Life Sci* 1963, 1, 73–79

Smith O W & G V S Smith The influence of diethylstilbestrol on the progress and out come of pregnancy as based on a comparison of treated with untreated primigravidas *Amer J Obstet Gynec* 1949, 58, 994–1009

Solomon, J B Relative growth of trophoblast and tumour cells co implanted into isogenic mouse testes and the inhibitory action of 'Methohexate' *Nature* 1966, 210, 716–718

Southam A L Historical review of intra uterine devices In Intra uterine contraception 2nd international conference October 2–3, 1964, New York Proceedings Amsterd 1965 pp 3–5

Staples, R E *Effect of clomiphene on blastocyst nidation in the rat Endocrinology* 1966, 78 82 86

Terner, N Ett nytt diphenylethylenderivat (F6066) Behandlingsresultat vid cancer pro statae *Nord Med* 1967, 77, 65

Ullberg S Autoradiographic studies on the distribution of labelled drugs in the body In International conference on the peaceful use of atomic energy 2 (Geneva 1958), Proceeding 24 248–254

Ullberg S & G Bengtsson Autoradiographic distribution studies with natural oestrogens *Acta endocr* (Kbh) 1963, 43, 75–86

Watnick, A S, J Gibson, M Vinegra & S Tolksdorf Ethynyl estradiol A potent orally *active contraceptive in rats Proc Soc exp Biol* 1964, 116, 343–347

Velardo I T & F L Hisaw Quantitative inhibition of progesterone by estrogens in development of deciduomata. *Endocrinology* 1951, 49, 530–537

Wexler B C Reproductive defects and abnormal progeny of rats treated with triparanol Prevention of abnormalities by ACTH *Endocrinology* 1964, 74, 64–78

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Introduction

Mercury and its compounds have attracted many investigators Substances having properties as potent poisons have always interested man, and together with many valuable applications in industry and medicine, this has rendered mercury and the mercurials an old and interesting history (ALMQUIST 1929, KING 1957)

Mercury seems to be almost ubiquitous in nature, as a rule in very small quantities (STOCK & CUCUEL 1934) It appears in different minerals in the earth crust and is also present in small amounts in the atmosphere (RANKAMA & SAHAMA 1950)

In Sweden a special problem of mercury as a substance contaminating the environment appeared in the 1950 s A decrease in population numbers of certain seed eating birds and their avian predators was observed by several ornithologists Large amounts of mercury were discovered in dead and intoxicated birds (BORG 1958, BORG *et al* 1966), and many signs pointed to the use of mercurials especially methyl mercury, for seed disinfection in agriculture as the origin of the mercury (BERG *et al* 1966, BORG *et al* 1965, 1969) The mercuric pesticides were even considered to threaten several avian species with total extermination (HANSSON 1965) Remarkably high concentrations of mercury were also found in the eggs of certain species (BORG et al 1965 1969) and experimentally a decreased or inhibited hatchability could be shown of eggs from birds fed methyl mercury (BORG *et al* 1965, TEJNING 1967 a) High concentrations of mercury were also demonstrated in eggs from hens fed mercury treated seed (TEJNING & VESTERBERG 1964 WESTÖÖ 1965 1966 a) In 1966 the use of alkyl mercurials as seed disinfectants was prohibited Instead the earlier used alkoxyalkyl mercurials came into use The regulations resulted in abruptly decreased mercury levels in many birds (BORG 1968, WANNTORP *et al* 1967) and an increased reproduction of many species was observed

Studies of birds from museum collections had shown that the feathers of predatory birds feeding from fish presented steadily increasing mercury content values from about 1890 and onwards (BERG *et al* 1966), which eliminated the seed dressings as the main source of the mercury It has been known for a long time that fresh water fishes often have a high mercury content as compared with other living organisms (STOCK & CUCUEL 1934, RAEDER & SNEKVIK 1941) but the values which were demonstrated in Swedish fresh water fish were

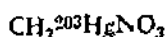
considerably above the older, "normal", values (WESTERMARK 1965, JOHNELS & WESTERMARK 1967) The use of mercury or mercurials in industry and to some extent in agriculture was considered to be the main source of the mercury contaminating the aquatic environment The paper, the pulp and the chlorine alkali industries were the dominating releasers of mercury contaminated waste waters There were also indications that mercury appeared as an airborne pollution affecting the aquatic environment The major part of the mercury in fish proved to be methyl mercury (WESTÖÖ 1966 b, NOREN & WESTÖÖ 1967) many times despite no methyl mercury, but inorganic or phenyl mercury, had been released in the water, and a conversion process was postulated (JENSEN & JERNELÖV 1969, HANNERZ 1968) The food hygienic risks illustrated by the experiences from the Minimata disease in Japan (KURLAND *et al* 1960), where many people were poisoned by methyl mercury contaminated fish resulted 1967 in a prohibition of the use of phenyl mercury in paper industry Fish from certain Swedish lakes were also judged unfit for human consumption (BERGLUND & WRETLIND 1967)

The present investigation was made in order to get more information on the distribution of mercury in the tissues of birds and fish A better knowledge of this might contribute to the understanding of the toxic effects of mercury and to the discussion of the potential risks for individuals in an environment contaminated with mercury The investigation has been made mainly using autoradiographic and scintillation counting techniques The distribution of mercury in the animals has been studied after administration of methyl phenyl methoxyethyl and inorganic mercury

In the avian experiments the Japanese quail (Coturnix coturnix japonica) has been used This species was chosen because of its hardiness ease of handling precocity great laying ability and small size making it available for whole body autoradiography Furthermore the quail is considered to be a suitable test animal for poultry in general (WILSON *et al* 1961) In the fish experiments pike (Esox lucius) perch (Perca fluviatilis) pike perch (Lucioperca lucioperca) salmon (Salmo salar) and speckled trout (Salvelinus fontinalis) have been used

CHAPTER ONE

Distribution of Methyl Mercuric Nitrate in the Quail



$CH_3{}^{203}HgNO_3$

Methyl mercurials were the most widely used compounds for seed disinfection in Sweden from their introduction at the beginning of the 1940's until they were prohibited by law in 1966. The symptoms in man poisoned by methyl mercury are well documented in toxicological literature (e.g. BIDSTRUP 1964) and the symptoms in birds poisoned with this compound were described by BORG *et al* (1965) and by TEJNING (1967 a).

In birds BORG (1958), SMART & LLOYD (1963) and BORG *et al* (1965) have reported mercury analyses of some organs after feeding the animals with methyl mercury. TEJNING & VESTERBERG (1964) studied the uptake of mercury in several organs and eggs from hens and TEJNING (1967 b) also studied the mercury contents of some organs of pheasants fed methyl and ethyl mercury dressed seed. In a more extensive study TEJNING (1967 a) examined the level of mercury in different organs of hens fed methyl mercury treated seed and he also studied the distribution in new hatched chicks. SWENSSON & ULFVARSON (1968 a) determined the mercury contents in some organs of poultry injected with methyl mercury and they also studied the rate of excretion.

In the present investigation whole body autoradiography (ULLBERG 1954, 1958) was performed on adult hen quails at different intervals after intravenous or peroral administration of the labelled mercurial. Eggs and chick embryos from the experimental birds were also autoradiographed. The distribution in some selected organs was studied by microautoradiographic technique (HAMMARSTRÖM *et al* 1965) and for this purpose cock quails were also used. Technical reasons made it necessary to pluck the birds and some feathers were autoradiographed separately. In addition to the autoradiographic work, impulse counting was made on some organs and eggs.

MATERIALS AND METHODS

Methyl mercuric nitrate labelled with ^{203}Hg and with a specific activity of 1.2 mCi/mg Hg was obtained from AB Atomenergi, Studsvik, Sweden. The compound was purified from inorganic mercury to a radiochemical purity of 98 % (ÖSTLUND 1969).

considerably above the older normal values (WESTERMARK 1965 JOHNELS & WESTERMARK 1967) The use of mercury or mercurials in industry and to some extent in agriculture was considered to be the main source of the mercury contaminating the aquatic environment The paper the pulp and the chlorine alkali industries were the dominating releasers of mercury contaminated waste waters There were also indications that mercury appeared as an airborne pollution affecting the aquatic environment The major part of the mercury in fish proved to be methyl mercury (WESTÖÖ 1966 b NOREN & WESTÖÖ 1967) many times despite no methyl mercury but inorganic or phenyl mercury had been released in the water and a conversion process was postulated (JENSEN & JERNELÖV 1969 HANNERZ 1968) The food hygienic risks illustrated by the experiences from the Minimata disease in Japan (KURLAND *et al* 1960) where many people were poisoned by methyl mercury contaminated fish resulted 1967 in a prohibition of the use of phenyl mercury in paper industry Fish from certain Swedish lakes were also judged unfit for human consumption (BERGLUND & WRETLIND 1967)

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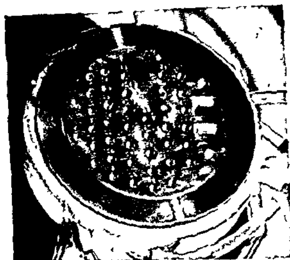


Fig 1 Sample holder of the freeze drying apparatus Copper plate with pits for frozen specimens chilled by thermoelements

xylene Finally they were embedded in paraffin Since the mercurial was water-soluble and a certain loss of radioactivity was observed during the preparation of the tissues small pieces were also freeze dried in order to control an eventual loss or redistribution of mercury in the fixation bath At this procedure the specimens were *rapidly frozen in isopentane cooled with liquid nitrogen* (—160° C) and then freeze dried in vacuum (10^{-4} mm Hg) for 5 days The freeze drying was performed in an apparatus which was built after original ideas of GLICK & MALMSTRÖM (1952), MOBERGER *et al* (1953) and PEARSE (1963) The frozen specimens were transferred to hollows drilled in a copper plate cooled to —32° C with three Peltier thermoelements (PT 20/20, Philips, Fig 1) The plate with the samples was placed as the outer bottom of a large glass thermos which was evacuated by means of a high vacuum pump (model 2SC 20 A) and an oil vapour diffusion pump (model 102A, Edwards High Vacuum Ltd, England) The inner container of the thermos was automatically filled with liquid nitrogen from a storage container and served as a first "cold trap When the freeze-drying was completed, the ice on the cold trap was sublimated to a second cold trap and the specimens were embedded in degassed paraffin previously placed in the hollows of the copper plate The paraffin was melted by reversing the current fed to the Peltier elements which then instead heated the plate When the formalin fixed or freeze dried specimens had been embedded 4 micron thick sections were taken on tape (No 688, Minnesota Mining and Manufacturing Company) The tape sections were mounted on G 5 nuclear plates (Ilford, 10μ) After exposure (20—100 days), the tape was removed in xylene, the film was developed and the section, still on the film, was stained with hematoxylin and eosin

The quails were purchased from a farm that had bred quails for many years ensuring that the birds were many generations removed from the wild The quails were kept in the laboratory in individual cages with wire floors and had a free access to drinking water They were fed a commercial pelleted pheasant ration containing only small amounts of mercury (10—20 ng/g) All day artificial lighting was always used during the experiments Incubation of eggs was performed in an incubator at 100° F and the humidity was kept at 65 %

Whole body autoradiography

Six hen quails each weighing about 150 g received the mercurial intravenously in a radial vein Three hens were also given the compound via a stomach tube

The dose calculated as mercury was in the first group 0 5 mg/kg body weight and in the other group 1 5 mg/kg The birds injected intravenously were sacrificed 10 minutes 1 hour 4 hours 1 day 4 days and 10 days after injection and the other birds after 1 day 4 days and 10 days respectively At the time of sacrifice the quails were anaesthetized with carbon dioxide plucked and immersed into a bath of hexane containing solid carbon dioxide (about —78° C)

After freezing the birds were embedded in carboxy methylcellulose mixed with water and mounted on a microtome stage in a freezing room (—17° C) Sagittal whole body sections were cut through the birds at different levels with a microtome Thirty micron thick sections were obtained by applying an adhesive tape (Minnesota Mining and Manufacturing Co No 810) to the section surface of the frozen bird before cutting After drying in the freezing room the sections were transferred to room temperature and autoradiograms were made by apposition of the sections against X ray film (Structurix Gevaert) In order to facilitate comparative studies between autoradiograms with different times of exposure and to make semiquantitative comparisons between different organs an isotope staircase of ^{203}Hg in two fold serial dilutions was applied to some of the films The isotope staircase was made according to the method described by BERLIN & ULLBERG (1963 I) After an exposure time of 10—80 days the sections were separated from the films The films were developed and the sections stained with hematoxylin and eosin and mounted on glass slides in Euparal®

Microautoradiography

Two male and two female quails were injected intravenously with methyl mercuric (^{203}Hg) nitrate The dose calculated as mercury was 1 0 mg/kg body weight One bird of each sex was sacrificed 1 and 7 days after injection and specimens were taken from various organs and fixed in buffered formalin (10 % pH 7 5) for 1—3 days The specimens were then passed through a series of increasing concentrations of ethanol (70 % 96 % and abs) and transferred to

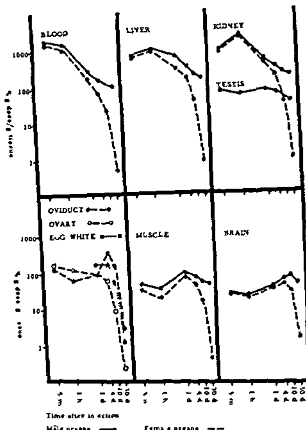


Fig. 2 Concentration of mercury in some tissues of quails various times after an i.v. injection of methyl mercuric nitrate. The ordinate is log₁₀ of the concentration per gram tissue as percentages of the dose given per gram animal. Each value represents the mean of 3 birds. Note the difference between the sexes and the delayed cerebral uptake.

During the first days after injection the cerebellar uptake dominated but after longer survival times there seemed to be a somewhat higher concentration in the cerebral hemispheres (Fig. 4). The concentration in the grey matter of the cerebellum was markedly higher than the concentration in the white matter and there was also more mercury in the granular than in the molecular layer. No special uptake was seen in the nerve cells. The uptake in the sympathetic and spinal ganglia was about equal to the body average. In the eye there was a moderate concentration of mercury in the choroid and in the retina. A marked accumulation was seen in the pecten and in the lens.

The digestive system

The resorption of ingested methyl mercury was rapid. One day after administration very little mercury could be detected in the digestive canal. A marked uptake and retention occurred in the epithelium of the mouth, the oesophagus and the crop. The bill concentrated mercury intensely (Fig. 3). The intestinal mucosa slightly accumulated mercury throughout its length. Up to 1 hour after injection

Impulse counting

In addition to the autoradiographic studies, quantitative analyses of the mercury contents in some organs was performed. Male and female quails were given a single intravenous injection of methyl mercuric (^{203}Hg) nitrate in water solution. The dose corresponded to 3 μg of mercury. Three birds of each sex were sacrificed 5 minutes, 1 hour, 1 day, 4 days, 10 days and 30 days after injection. Samples were taken of blood, liver, kidney, muscle, brain, testis, ovary, oviduct and eventual eggs. The samples were weighed and analysed for radioactivity in a gamma spectrometer (Packard). The values obtained were calculated to give the concentration of radioactivity per gram of the tissue as percentages of the injected dose per gram body weight.

The eggs from two groups of hen quails were also sampled and analysed for mercury. Each hen in the first group (3 hens) received a dose corresponding to 4 μg of mercury intravenously and each hen in the second group (2 hens) was injected with a dose representing 16 μg of mercury. Yolk and white of the eggs were separated, weighed, homogenized and analysed for mercury. The values obtained were calculated to give the total mercury contents of each egg as percentages of the injected dose and the concentration per gram yolk and white as percentages of the injected dose per gram body weight.

RESULTS

The distribution of mercury was essentially the same, no matter how the methyl mercury was administered. Resorption in the digestive canal was very good. With the exception of a few organs the mercury was distributed very uniformly in the body. In the following the distribution in the various tissues will be described in detail. Unless something else is said, the description refers to the quails injected intravenously.

The circulatory system

The mercury concentration in the circulating blood decreased very slowly with time (Fig. 2—4). A pronounced difference appeared between the sexes, the concentration in the blood of the cocks falling more slowly than that in the blood of the hens (Fig. 2). The myocardium initially showed a marked uptake of mercury but 1 day after injection and later the concentration was about equal to that in most other organs. The bone marrow, the spleen and the thymus presented a moderate concentration throughout the study.

The nervous system

The brain and the spinal cord showed an increasing accumulation of mercury during the first days after injection (Fig. 2). The maximum concentration was observed on the 10th day after injection in the cocks whereas the hens had reached a maximum after 4 days. The mercury distributed rather uniformly in the brain



Fig 4 Distribution of mercury in a quail 10 days after an i v injection of methyl mercuric nitrate

the concentration in the proventriculus was a little higher than in the gizzard but at longer survival times there was no difference After ingestion a high concentration of mercury appeared in the koilin layer of the gizzard The liver presented a moderate uptake of mercury (Fig 4) and a rather slow elimination especially in the cocks Mercury appeared *in the gall bladder 1 hour after injection* and was still visible 10 days later In the pancreas a rather high concentration was seen shortly after injection but later it was about the same as the body average (Fig 4) In the salivary glands moderate amounts of mercury were seen up to four days after injection

The endocrine system

No remarkable accumulation exceeding the average body concentration occurred in the endocrine organs In the thyroid and in the adrenals there was a moderate uptake with an even distribution The endocrine pancreas showed a concentration somewhat lower than its exocrine part The pituitary accumulated mercury to the same concentration as the brain The distribution seemed to be even between the various cells of the adenohypophysis

The urinary system

The kidneys reached a high concentration of mercury shortly after injection The concentration then slowly decreased more rapidly in the hens than in the cocks (Fig 2) On the microautoradiograms the mercury appeared evenly distributed in most parts of the nephrons Only the collecting tubules had a concentration markedly lower than the other parts No differences were seen between the formalin fixed and freeze-dried specimens In the urine only minute amounts of mercury could be detected

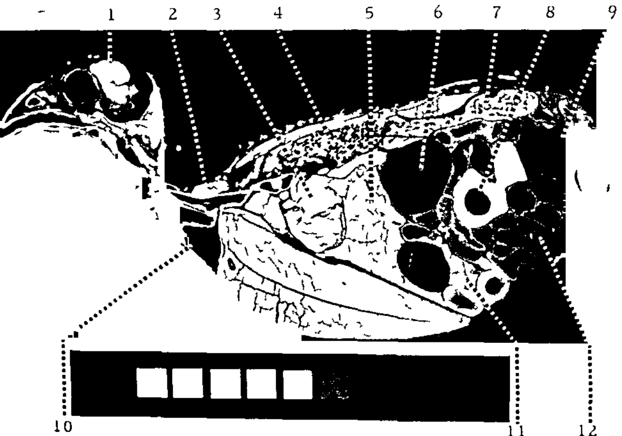


Fig 3 Distribution of mercury (white areas) in a quail 4 days after an i v injection of methyl mercuric nitrate An isotope staircase has been placed below the autoradiogram
1 brain 2 thymus 3 heart blood 4 lung 5 liver 6 follicular yolk 7 kidney 8 yolk in magnum of oviduct 9 uterine part of oviduct 10 crop 11 pancreas 12 intestines



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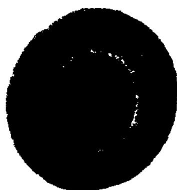


Fig 5 Autoradiogram of an egg laid by a quail injected with methyl mercuric nitrate Note the selective accumulation of mercury in the albumen The time of injection is represented by the light ring in the yolk Successive layers of yolk show a very faint uptake of mercury

The reproductive system

The ovarian stroma and the follicular cells showed a moderate uptake of mercury Growing follicles presented a slight uptake of mercury in a narrow zone developed during the hours following the injection The succeeding layers of yolk contained very little or no mercury (Fig 5) After ingestion of the mercurial the zone was not observed and the yolk contained only very small amounts of mercury In the germinal disc a moderate uptake of mercury was seen A very pronounced accumulation occurred in the oviduct (Fig 3 39) This was obvious 1 day after injection and showed its maximum after 4 days The retention was very prolonged and at the longest survival periods the mercury concentration in the oviduct dominated over all the other organs and was comparable only with the concentration in the feathers and in the albumen The largest amounts appeared in the albumen producing magnum and less was seen in other parts The mercury localized evenly in the tubular glands of the mucosa and very little was seen in the surface epithelium or in the mucoid secreting cells The concentration in the oviduct was manifested by a strong excretion of mercury in the white of the eggs (Fig 4—6) No difference was observed between the various layers of thick and thin albumen At the quantitative measurements about 50 % of the injected dose was excreted via the eggs, mainly during the first week

Table 1 Excretion of mercury in the eggs of three quails (A, B C) injected intravenously with a single dose of methyl mercuric nitrate (4 μgHg) The accumulation of mercury in the albumen permits a large amount of the injected dose to be excreted via the eggs, mainly during the first week after injection

Day after injection	% of injected dose in eggs A	B	C
1	0 01	0 02	0 02
2	7 25	11 64	9 26
3	11 81	10 84	9 83
4	8 32	—	6 96
5	5 74	7 62	—
6	3 79	5 24	3 44
7	3 02	3 91	2 42
Excreted in eggs during 1 st week	39 94	39 27	31 93
8	2 36	2 85	—
9	—	2 36	1 81
10	1 86	1 76	1 30
11	1 42	1 37	0 98
12	1 61	1 50	1 04
13	0 92	—	0 72
14	0 67	0 90	0 56
2nd week	8 84	10 72	6 41
3rd week	1 34	2 36	1 72
4th week	0 21	0 50	0 33
Total	50 33	53 05	40 39

after injection (Table 1) The highest mercury values were seen in the eggs laid on the third day No greater difference in the egg excretion of mercury was seen depending on the dose injected In the shell membranes very little, and in the shell no mercury accumulated

The testes accumulated moderate amounts of mercury which localized in the interstitial tissue as well as in the seminiferous epithelium The activity over areas with aggregated spermatozoas did not exceed the average in the tubuli The epididymis showed a concentration of mercury equal to that of the testes. The same concentration of mercury was also seen in the seminal fluid

Others

The lungs reached a high concentration of mercury immediately after injection At longer survival times or in the birds given methyl mercury perorally, the concentration did not exceed that of most other organs

The skeletal muscles accumulated considerable amounts of mercury Some muscles e g the superficial pectoral concentrated more than others No mercury

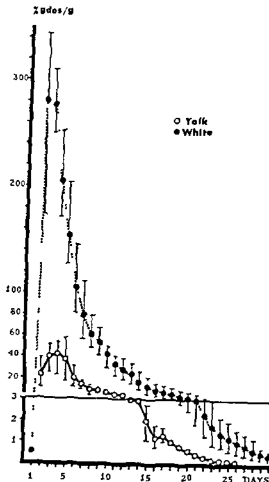


Fig 6 Concentration of mercury in yolk and egg white in eggs from quails injected i v with methyl mercuric nitrate The ordinate (two scales) is $\log_{10}$ of the concentration per gram yolk or white as percentages of the dose given per gram animal Each value represents two or three eggs the range is indicated by vertical lines

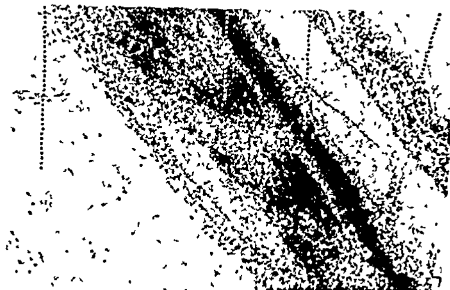
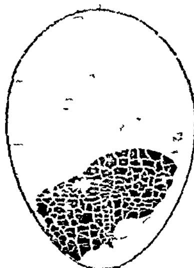


Fig 7 Microautoradiogram of a contour feather showing the accumulation of methyl mercury (black grains) in the barb ridges in the zone of keratinization and in the protective sheath





F g 8 Autorad ogram (left) and correspond ng section of an egg from a qua l injected w th methyl mercuric n trate The egg has been incubated for 10 days The mercury is localized n the albumen and no mercury s seen in the yolk or in the embryo





F g 9 Aut ad grams of a methyl mercury-conta n ng egg incubated for 15 days The mer ury has been transferred from the albumen sac (Cf above) to the yolk sac. A pronounced uptake f mercury is also seen in the embryo

1 bra n 2 lung 3 external yolk sac 4 internal yolk sac 5 g zzard 6 l ver 7 heart 8 proventr culus 9 k dney

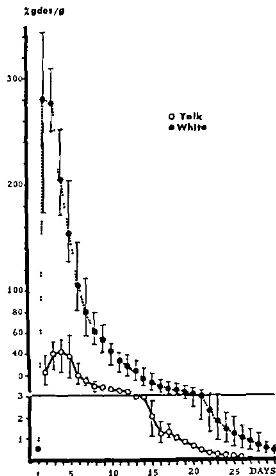


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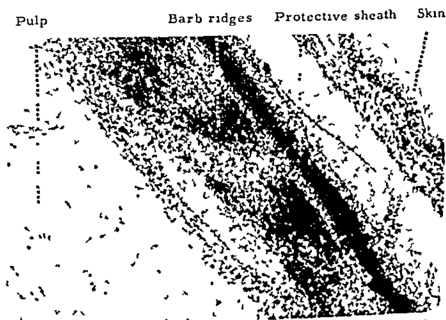
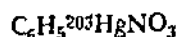


Fig 7 Microautoradiogram of a contour feather showing the accumulation of methyl mercury (black grains) in the barb ridges in the zone of keratinization and in the protective sheath

Distribution of Phenyl Mercuric Nitrate in the Quail



Phenyl mercurials were the first organic mercury compounds used as seed disinfectants (HUNTER 1955 ULFVARSON 1962) In Swedish agriculture *they were used during the* 1930 s and the 1940 s whereupon they were replaced by the alkylmercurials Poisoning of man or animals by this type of mercurial is very little mentioned in literature Anemia leucopenia and liver damage were described by COTTER (1947) *Neurasthenic symptoms* have also been reported (MASSMAN 1957) In experimental poisoning of mice the major symptoms derived from the gastro intestinal tract and the central nervous system (LUNDGREN & SWENSSON 1950) *In animal experiments nephrosis has also been* described (WEED & ECKER 1933)

The distribution of mercury in mammals after administration of phenyl mercury has been described by most investigators to be very similar to that seen after administration of inorganic mercury (Hg^{2+}), though certain differences have been noted (BERLIN & ULLBERG 1963 II, SWENSSON *et al* 1959 PRICKETT *et al* 1950) Very few investigations have been made using birds MILLER *et al* (1960) studied the uptake of mercury in chicks after intramuscular injections or ingestion of phenyl mercuric acetate MILLER *et al* (1959 a b 1967) also studied the uptake of phenyl mercury in different strains of chickens and could demonstrate a considerable difference in the retention of mercury SWENSSON & ULFVARSON (1968 a) studied the distribution in some organs and the excretion of mercury in white leghorn cocks after injection of phenyl mercuric hydroxide, and found the highest concentrations in the liver and in the kidneys

In the present investigation the previously described autoradiographic technique was used to study the distribution of mercury in the quail after administration of phenyl mercuric nitrate labelled with ^{203}Hg Scintillation counting was used to get a more quantitative information, and the excretion of mercury via the eggs was also studied

MATERIALS AND METHODS

^{203}Hg labelled phenyl mercuric nitrate with a specific activity of 1 2 mCi/mg mercury was obtained from AB Atomenergi Studsvik, Sweden The radiochemical purity was about 95 % the impurities consisting of inorganic mercury and

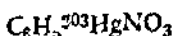
was seen in bone and very little in connective tissue In the subcutaneous fat depots small amounts appeared 1 hour after injection but at longer survival times no accumulation could be seen The concentration in the Bursa of Fabricius was quite low at all the times studied No mercury was observed in the glycogen body or in the uropygial gland

A very pronounced accumulation of mercury occurred in all parts of growing feathers Fully developed feathers which had started their growth after the time of injection, showed an uptake of mercury which only slightly decreased towards the quill (Fig 38) As a rule, no mercury was seen in parts keratinized before the mercurial was given In some feathers, however, a faint uptake was seen in earlier developed parts of the vane Microautoradiography of a developing contour feather showed the mercury to concentrate in the barb ridges and in the shaft in the zone of keratinization (Fig 7) A high concentration also appeared in the horny protective sheath Almost no mercury was seen in the dermal papilla the germinative zone or in the zone of differentiation

Incubated eggs

Very little mercury was seen in the fetuses in eggs incubated for 10 days (Fig 8) After an incubation period of 15 days, however, the chick fetuses had accumulated considerable amounts of mercury The concentration in the yolk sac, which previously had been low, was now high (Fig 9) The distribution of mercury in the chick was similar to that in the mother High concentrations were seen in the yolk sac, bill, feather follicles, scales of the tarsus and in the contents of some parts of the intestine The liver also showed a rather high uptake Moderate amounts appeared in the blood No mercury was observed in the bile The uptake in the kidneys was somewhat lower than in the liver The brain had a concentration about equal to that of the skeletal muscles

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$C_6H_5{}^{203}HgNO_3$

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Fig 11 Distribution of mercury (light areas) in a hen quail 10 minutes after an i v injection of phenyl mercuric nitrate
1 thyroid 2 lung 3 proventriculus 4 adrenal 5 ovary 6 kidney 7 oviduct 8 uropygial gland 9 heart blood 10 liver 11 spleen 12 gizzard 13 intestines



Fig 12 Distribution of mercury in a hen quail 1 day after an i v injection of phenyl mercuric nitrate
1 thyroid 2 lung 3 follicle accumulating yolk 4 kidney, 5 pancreas

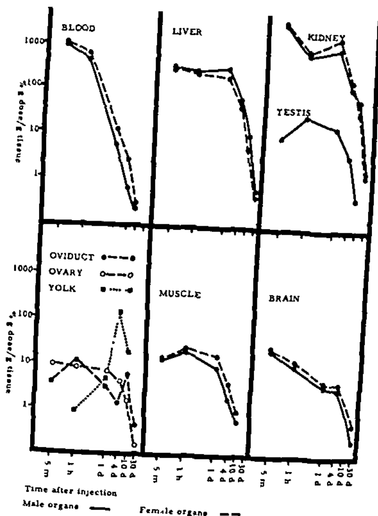


Fig 10 Concentration of mercury in some tissues of quails various times after a single i v injection of phenyl mercuric nitrate Each value represents the mean of 3 birds

diphenyl mercury The compound was dissolved in 40 % ethanol and administered to adult quails according to the plan described in the previous chapter The intravenous dose, calculated as mercury, was 0 5 mg/kg body weight and the peroral dose corresponded to 1 0 mg/kg The birds used at the quantitative measurements were injected intravenously with a dose corresponding to 4 µg of mercury At the studies of the mercury excretion via the eggs 3 hen quails were given 4 µg of mercury and 2 hens 16 µg

RESULTS

A more differentiated distribution of mercury than after administration of methyl mercury was seen, and the retention in various organs was less prominent Though, one day after ingestion, large amounts of mercury persisted in the alimentary canal, much mercury had distributed in the body



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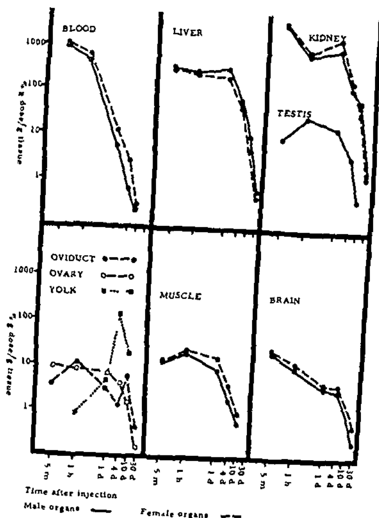


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Fig 13 Distribution of mercury in a hen quail 10 days after an i v injection of phenyl mercuric nitrate
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The circulatory system

The blood concentration of mercury decreased slowly, but more rapidly than after injection of methyl mercury (Fig 10) More mercury was retained in the blood of the females than in the blood of the males The sex difference was however less prominent than after injection of methyl mercury At the shortest survival times the concentration in the myocardium exceeded that in the skeletal muscles, but after one day no difference could be seen One day after injection the concentration in the spleen exceeded that in the blood (Fig 12) but after longer survival times there was no retention

The nervous system

A slight accumulation of mercury could be seen in the brain The delayed uptake, observed after injection of methyl mercury could, however, not be demonstrated, but the total contents of mercury in the brain steadily decreased (Fig 10) In the olfactory and optical lobes the concentration was somewhat higher than in the rest of the fore brain The spinal cord, the brain stem and the cerebral



Fig 14 Detail from an autoradiogram of a hen quail 10 days after an i v injection of phenyl mercuric nitrate Note the accumulation of mercury in the germinal disc and in the nucleus of Pander Alternating layers with varying concentrations appear in the yolk

hemispheres presented a somewhat higher uptake than the cerebellar cortex At the longer survival times the white matter of cerebellum showed a higher concentration of mercury than the grey matter

The digestive system

The glandular stomach presented a slight accumulation of mercury up to one day after injection No mercury, however, was seen in the contents of either the glandular stomach or the gizzard In the gizzard some mercury appeared in the muscle layer The koilin layer concentrated large amounts of mercury after ingestion Ten days after the intravenous injection, the koilin lining also showed a narrow zone with a marked uptake (Fig 13).

The intestinal mucosa presented a moderate concentration of mercury shortly after injection (Fig 11) Mercury also appeared in the intestinal contents With increasing survival time of the birds, the intestinal mucosa increased its uptake of mercury (Fig 12) More mercury also appeared in the lumen The liver presented a very high accumulation of mercury, the highest concentration appearing one day after injection (Fig 10, 12) At this time much mercury was also seen in the bile After ingestion of phenyl mercury the liver concentration seemed



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Fig 16 Autoradiograms of two 15 days old *fetuses from* eggs of quails injected with phenyl mercuric nitrate High concentrations *of mercury* appear in yolk sac, liver, gastro intestinal tract and Wolffian body
1 yolk, 2 liver, 3 Wolffian body, *4 kidney*, 5 gizzard, 6 proventriculus

after *injection*, but after longer survival periods only small amounts *persisted. No mercury* was observed in the *glycogen body (Fig* 13). *In* growing feathers there was a strong uptake. The concentration was highest in parts formed shortly *after injection*, and in subsequently *developed* parts the concentration gradually decreased *In the* region of the upper navel (umbilicus superior) *many feathers* showed an intense accumulation of mercury in a membranous collar *surrounding* the quill *High* concentrations were also seen in the membrane forming the upper navel

Incubated eggs

The chick fetuses accumulated mercury from the earliest stages of *development* The highest concentration was seen in the internal yolk sac (Fig 16) The liver and the gall bladder showed relatively high concentrations while less *activity was seen* in the kidneys A pronounced accumulation occurred in the periphery of the vertebrae A high uptake was also observed in the posterior part of the *Wolffian body (mesonephros)*, where the mercury seemed to localize in the walls of the tubules Small amounts of mercury could be detected in the skeletal muscles and in the central nervous system

Distribution of Methoxyethyl Mercuric Hydroxide in the Quail



$CH_3OC_2H_4{}^{203}HgOH$

The search for effective weapons in the fight against plant diseases among many others has presented the alkoxyalkyl mercurials These have earlier been used in Swedish agriculture and were reintroduced when the alkylmercurials were *prohibited* by the authorities in 1966 In other countries, however, these compounds have been used for a long time Our knowledge of these mercurials as regards their toxicity and distribution in man and animals is rather limited Their acute toxicity *is considered to be of the same* order as that of the alkyl or aryl mercurials (HAGEN 1955, SWENSSON & ULFVARSON (1963) The methoxyethyl mercuric ion is chemically unstable and is rapidly decomposed in soil and *in animal tissues (BOOER 1944, ULFVARSON 1962, SWENSSON &* ULFVARSON 1968 a) Distribution and excretion studies of methoxyethyl mercury have earlier been performed on rats (ULFVARSON 1962), poultry (SWENSSON & ULFVARSON *1968 a) and on pheasants (HELMINEN et al* 1966) The kidneys and the liver are the main target organs and the compound is rapidly excreted A pronounced accumulation also occurs in the feathers and in the ovary

Poisoning of man by alkoxyalkyl mercury has been described (ZEYER 1952), but feeding of poultry, swine and horse with methoxyethyl mercuric silicate did not result *in any toxic symptoms (ROTHES & HAVERMANN* 1939) Neither could HELMINEN *et al* (1966) observe any serious toxic effects when they fed the compound to pheasants

In the present investigation the distribution of methoxyethyl mercuric hydroxide in quail has been studied by whole body autoradiographic and scintillation technique The distribution in the pituitary was also examined by a histochemical method

MATERIALS AND METHODS

Methoxyethyl mercuric hydroxide labelled with ^{203}Hg and with a specific activity of 400 μCi/mg mercury was obtained in water solution from AB Atomenergi, Studsvik Sweden The radiochemical *purity was > 90 % Adult hen* quails were administered the compound according to the plan described in chapter one Two

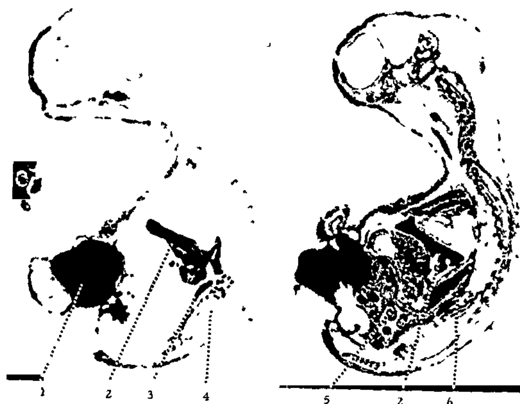


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1 yolk, 2 liver, 3 Wolffian body, 4 kidney, 5 gizzard, 6 proventriculus

after injection, but after longer survival periods only small amounts persisted No mercury was observed in the glycogen body (Fig 13) In growing feathers there was a strong uptake The concentration was highest in parts formed shortly after injection, and in subsequently developed parts the concentration gradually decreased In the region of the upper navel (umbilicus superior) many feathers showed an intense accumulation of mercury in a membranous collar surrounding the quill High concentrations were also seen in the membrane forming the upper navel

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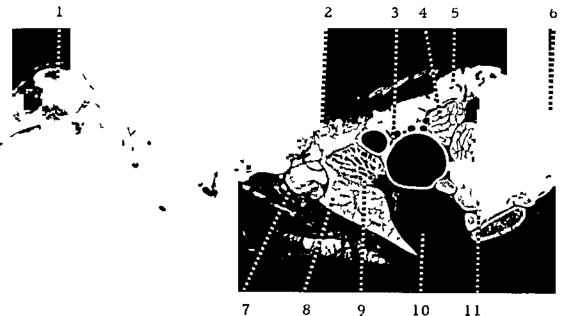


Fig 18 Whole body autoradiogram of a hen quail 4 hours after an i v injection of methoxyethyl mercuric hydroxide The dominating uptake of mercury (light areas) is seen in the kidneys A high concentration is also seen in the peripheral layers of follicular yolk and in the magnum of the oviduct
1 brain 2 lung 3 ovary 4 oviduct (magnum) 5 kidney 6 egg 7 heart blood 8 liver 9 proventriculus 10 gizzard 11 intestines

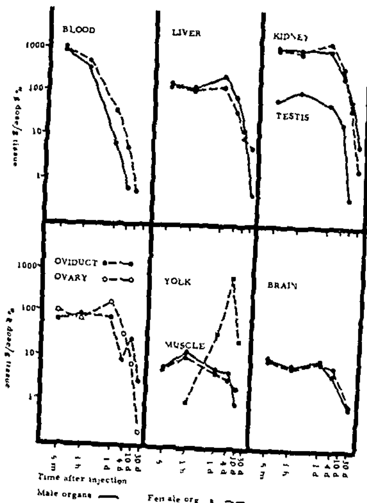


Fig 17 Concentration of mercury in some tissues of quails various times after a single iv injection of methoxyethyl mercuric hydroxide Each value represents the mean of three birds

cocks were also injected intravenously and autoradiographed after 1 and 10 days respectively The parenteral dose at the autoradiographic work corresponded to 1 0 mg of mercury per kg body weight and at the quantitative work to 1 μg per bird At the studies of the egg excretion two hens were also injected with 16 μg of mercury Whole body autoradiography and impulse counting of organs was performed as previously described The localization of mercury in the pituitary was also demonstrated histochemically by the sulphide silver method (TIMM 1958 VOIGT 1962) A hen quail was injected intramuscularly with methoxyethyl mercuric hydroxide (non labelled) corresponding to 6 mg of mercury The dose was dispersed and administered as daily injections during 4 days in succession The bird was sacrificed 10 days after the first injection was given and the pituitary was fixed in H_2S saturated alcohol (70 %) After fixation, the specimen was embedded in paraffin and sectioned The sections were treated for 15 minutes with a 15 % aqueous solution of hydrogen peroxide and thereafter physically developed with silver nitrate and hydroquinone



Fig 20 Distribution of mercury in a hen quail 4 days after an i v injection of methoxyethyl mercuric hydroxide.
1 brain 2 adrenal 3 kidney 4 yolk in oviduct 5 heart 6 liver 7 spleen 8 oviduct, 9 pancreas.

like that seen after injection of methyl mercury could be demonstrated A pronounced uptake of mercury was also seen in sympathetic and spinal ganglia (Fig 21)

The digestive system

The bill and the epithelium of the tongue presented a high uptake of mercury In the oesophageal glands a large concentration was seen throughout the study A very high uptake of mercury was seen in the proventriculus of the cock which was autoradiographed 1 day after the injection (Fig 21) A similar uptake was never seen in the hens neither after injection nor after peroral dosage A pronounced accumulation of mercury appeared in the intestinal mucosa (Fig 21 22) The intestinal contents showed high concentrations 1 hour after injection and at the same time a considerable amount also appeared in the bile (Fig 23)

Moderate amounts of mercury accumulated in the liver which due to a higher uptake in the portal tracts got a marbled appearance (Fig 22)

The exocrine pancreas presented a very pronounced uptake and a strong retention of mercury when the compound was injected (Fig 19 20 22) Peroral dosage resulted in a less marked uptake.

The urinary system

The kidneys presented the highest uptake of mercury of all organs studied (Fig 17—22) In the renal lobules the highest concentration occurred in the cortical tubules and in straight tubules radiating towards the papilla and probably identical with the straight portions of the proximal tubules In the ureters only small amounts of mercury could be observed

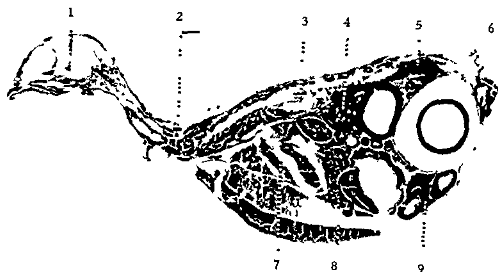


Fig 19 Distribution of *mercury in* a hen quail 1 day after an i v injection of methoxyethyl mercuric hydroxide

1 pituitary 2 *thymus*, 3 lung 4 ovary, 5 kidney, 6 uropygial gland, 7 heart 8 liver, 9 pancreas

RESULTS

The peroral dose was badly *resorbed One* day after ingestion large amounts *persisted in the* digestive tract and high concentrations appeared in the intestinal *mucosa* and in the koilin lining of the gizzard Because no greater differences in *the distribution* of mercury due to the way of administration were observed *the following* mainly accounts for the birds *injected intravenously*

The circulatory system

The blood concentration declined rather slowly The mercury content *values in the blood of* the cocks seemed to decrease more rapidly than in the blood of the hens (Fig 17) The initially high concentration in the myocardium soon decreased but a slight retention could be observed at the longer survival times The bone marrow also had a high concentration of mercury shortly after injection which *then gradually* declined with about the same speed as the blood concentration The spleen accumulated moderate amounts of mercury up to 4 days after injection The white pulp seemed to concentrate more mercury than the red pulp (Fig 20) The thymus showed a marked retention of mercury even at the longer survival times

The nervous system

The brain and the spinal cord showed a small uptake of mercury with a relatively even distribution A high concentration was observed in the choroid plexa in the olfactory lobe and in some brain nuclei (Fig 21) No overall accumulation



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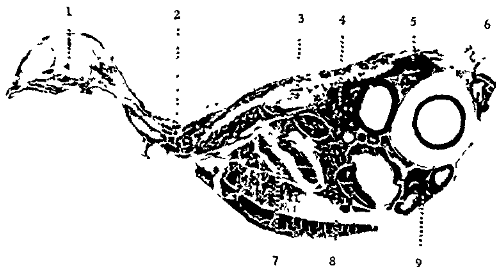


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The peroral dose was badly resorbed One day after ingestion large amounts persisted in the digestive tract and high concentrations appeared in the intestinal mucosa and in the koilin lining of the gizzard Because no greater differences in the distribution of mercury due to the way of administration were observed the following mainly accounts for the birds injected intravenously

The circulatory system

The blood concentration declined rather slowly The mercury content values in the blood of the cocks seemed to decrease more rapidly than in the blood of the hens (Fig 17) The initially high concentration in the myocardium soon decreased, but a slight retention could be observed at the longer survival times The bone marrow also had a high concentration of mercury shortly after injection which then gradually declined with about the same speed as the blood concentration The spleen accumulated moderate amounts of mercury up to 4 days after injection The white pulp seemed to concentrate more mercury than the red pulp (Fig 20) The thymus showed a marked retention of mercury even at the longer survival times

The nervous system

The brain and the spinal cord showed a small uptake of mercury with a relatively even distribution A high concentration was observed in the choroid plexa in the olfactory lobe and in some brain nuclei (Fig 21) No overall accumulation

A considerable part of the administered dose could be recovered in the eggs The larger dose injected resulted as well totally as relatively in an increased excretion via the eggs (Table 2 3) The major part of the mercury localized in the yolk (Fig 28) Ten minutes after injection a high concentration was visible in the wall of ovarian follicles accumulating yolk, and 1 hour later a narrow zone with an intense uptake of mercury could be seen in the periphery of the yolk (Fig 18) Ten days after injection the entire yolk had accumulated mercury The previously described varying uptake in white and yellow yolk was also observed In some eggs the gradual decrease in mercury concentration of the strata of yellow yolk suddenly was interrupted by an increase Occasionally atretic follicles with a high concentration of mercury were observed There was, however, no indication of more atretic follicles in these ovaries than are normally present In some cases a bursting atresia had resulted in a high concentration of mercury in the ovarian interstitium. No germinal discs were identified

Day after injection	% of injected dose in eggs A	B
1	—	—
2	0 37	—
3	3 10	3 99
4	—	10 06
5	17 84	—
6	15 61	19 06
7	13 16	17 63
Excreted in eggs during 1 st week	50 08	50 74
8	6 99	3 63
9	5 26	—
10	1 55	—
11	—	2 29
12	1 34	1 43
13	0 72	0 89
14	0 47	0 57
2nd week	16 33	8 81
3rd week	1 34	1 41
4th week	0 35	0 30
Total	68 10	61 26

Table 3 Excretion of mercury in the eggs of two hen quails (A B) injected intravenously with a single dose of methoxyethyl mercuric hydroxide corresponding to 16 µg of mercury The increased dosage resulted in a relatively larger excretion of mercury

The thyroid slightly accumulated mercury with an even distribution during the first days after injection The concentration in the parathyroid was more pro nounced than in the thyroid (Fig 24) The medullary cells of the adrenals showed a high concentration of mercury from day 1 and onwards Less activity was seen in the interrenal cells (Fig 27)

The anterior pituitary presented a strong accumulation and a prolonged reten tion of mercury (Fig 22) The caudal lobe showed a stronger uptake than the cephalic lobe (Fig 25) Ten days after injection the concentration in the caudal lobe was comparable with, or even exceeded that in the kidneys The histo chemical visualization of mercury showed it to localize in different types of cells in the adenohypophysis Certain cells contained considerably more than the others These cells stained red with azan and contained a large, pale nucleus (Fig 26) The mercury was localized only in the cytoplasm and could not be demonstrated in the nucleus In the neurohypophysis no mercury could be observed

Day after injection	% of injected dose in eggs A	B	C
1	0 01	—	—
2	1 10	—	0 10
3	—	1 28	3 32
4	3 56	—	—
5	—	4 48	—
6	5 26	3 18	6 25
7	4 67	2 79	5 04
Excreted in eggs during 1 st week	14 60	11 73	14 71
8	2 51	1 43	2 85
9	—	1 05	—
10	0 96	—	2 04
11	0 71	0 63	0 82
12	0 49	0 26	0 53
13	0 25	0 23	0 40
14	0 17	0 18	0 38
2nd week	5 09	3 78	7 02
3rd week	0 44	0 56	0 55
4th week	0 06	0 13	0 01
Total	20 19	16 20	22 29

Table 2 Excretion of mercury in the eggs of three hen quails (A B C) injected intravenously with a single dose of methoxy ethyl mercuric hydroxide corre sponding to 4 μg of mercury

The reproductive system

A considerable part of the administered dose could be recovered in the eggs The larger dose injected resulted as well totally as relatively in an increased excretion via the eggs (Table 2, 3) The major part of the mercury localized in the yolk (Fig 28) Ten minutes after injection a high concentration was visible in the wall of ovarian follicles accumulating yolk, and 1 hour later a narrow zone with an intense uptake of mercury could be seen in the periphery of the yolk (Fig 18) Ten days after injection the entire yolk had accumulated mercury The previously described varying uptake in white and yellow yolk was also observed In some eggs the gradual decrease in mercury concentration of the strata of yellow yolk suddenly was interrupted by an increase Occasionally atretic follicles with a high concentration of mercury were observed There was, however, no indication of more atretic follicles in these ovaries than are normally present In some cases a bursting atresia had resulted in a high concentration of mercury in the ovarian interstitium No germinal discs were identified

Day after injection	% of injected dose in eggs A	B
1	—	—
2	0 37	—
3	3 10	3 99
4	—	10 06
5	17 84	—
6	15 61	19 06
7	13 16	17 63
Excreted in eggs during 1 st week	50 08	50 74
8	6 99	3 63
9	5 26	—
10	1 55	—
11	—	2 29
12	1 34	1 43
13	0 72	0.89
14	0 47	0 57
2nd week	16 33	8.81
3rd week	1 34	1 41
4th week	0 35	0 30
Total	68 10	61 26

Table 3 Excretion of mercury in the eggs of two hen quails (A B) injected intravenously with a single dose of methoxyethyl mercuric hydroxide corresponding to 16 µg of mercury The increased dosage resulted in a relatively larger excretion of mercury

The endocrine system

The thyroid slightly accumulated mercury with an even distribution during the first days after injection The concentration in the parathyroid was more pronounced than in the thyroid (Fig 24) The medullary cells of the adrenals showed a high concentration of mercury from day 1 and onwards Less activity was seen in the interrenal cells (Fig 27)

The anterior pituitary presented a strong accumulation and a prolonged retention of mercury (Fig 22) The caudal lobe showed a stronger uptake than the cephalic lobe (Fig 25) Ten days after injection the concentration in the caudal lobe was comparable with, or even exceeded that in the kidneys The histochemical visualization of mercury showed it to localize in different types of cells in the adenohypophysis Certain cells contained considerably more than the others These cells stained red with azan and contained a large, pale nucleus (Fig 26) The mercury was localized only in the cytoplasm and could not be demonstrated in the nucleus In the neurohypophysis no mercury could be observed

Day after injection	% of injected dose in eggs A	B	C
1	0 01	—	—
2	1 10	—	0 10
3	—	1 28	3 32
4	3 56	—	—
5	—	4 48	—
6	5 26	3 18	6 25
7	4 67	2 79	5 04
Excreted in eggs during 1 st week	14 60	11 73	14 71
8	2 51	1 43	2 85
9	—	1 05	—
10	0 96	—	2 04
11	0 71	0 63	0 82
12	0 49	0 26	0 53
13	0 25	0 23	0 40
14	0 17	0 18	0 38
2nd week	5 09	3 78	7 02
3rd week	0 44	0 56	0 55
4th week	0 06	0 13	0 01
Total	20 19	16 20	22 29

Table 2 Excretion of mercury in the eggs of three hen quails (A B C) injected intravenously with a single dose of methoxyethyl mercuric hydroxide corresponding to 4 µg of mercury



Fig 22 Localization of mercury in a cock quail 10 days after an i v injection of methoxy ethyl mercuric hydroxide *Note the retention of mercury in the anterior pituitary and in the pancreas*



Fig 23 Detail from an autoradiogram of a hen quail 1 hour after an i v injection of methoxyethyl mercuric hydroxide *A high concentration of mercury is seen in the bile*



Fig 24 Detail from an autoradiogram of a hen quail 1 day after an i v injection of methoxyethyl mercuric hydroxide *Note the concentration of mercury in the parathyroid*

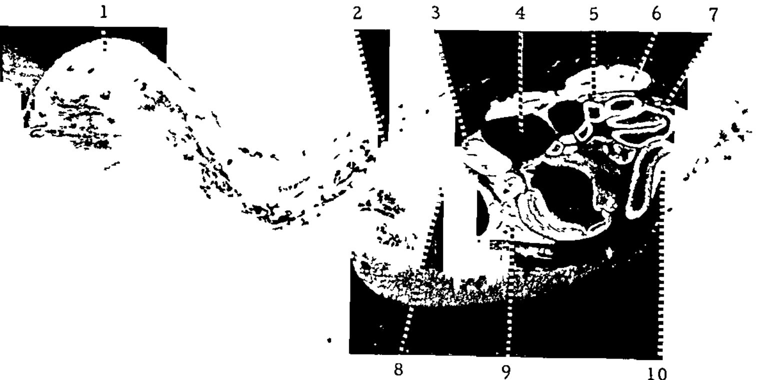


Fig. 21 Localization of mercury in a cock quail 1 day after an i.v. injection of methoxyethyl mercuric hydroxide. Note the accumulation of mercury in the pituitary, in the kidney and in the pancreas. Only a slight uptake is seen in the testes whereas the epididymis shows a strong uptake. A high uptake is also seen in spinal and sympathetic ganglia. The strong concentration of mercury seen in the proventriculus never appeared in the hen quails. 1 brain with pituitary, 2 ganglia, 3 proventriculus, 4 testis, 5 epididymis, 6 kidney, 7 intestine, 8 heart blood, 9 liver, 10 pancreas

Fig 26 Histochemical visualization of mercury (black grains see arrows) in a quail anterior pituitary 10 days after an i v injection of methoxyethyl mercuric hydroxide Certain cells, with a large pale nucleus and a large nucleolus, accumulate more mercury in their cytoplasm than other cells

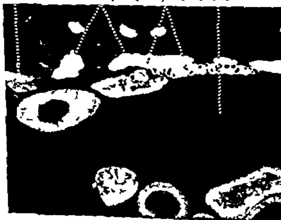


Fig 27 Detail from an autoradiogram of a cock quail 10 days after i v injection of methoxyethyl mercuric hydroxide Note the retention of mercury in the epididymis and in the adrenal medullary cells



Fig. 25 Autoradiogram (top) and corresponding section of the head of a hen quail 10 days after an i.v. injection of methoxyethyl mercuric hydroxide. A pronounced retention of mercury is seen in the anterior pituitary. The caudal lobe has a higher concentration than the cephalic lobe.

BRAIN

KIDNEY UROPYGIAL GLAND

EGG WHITE EGG YOLK

ı of mercurv (light areas) ın a hen quail 4 days after an ı v injection ›ote the accumulation of mercury ın kidney yolk and pituitary A rup ad mercury containing yolk ın the abdominal cavity

tıc and spinal ganglia A high concentration was observed in d in some cranial nerves, probably N trigeminus and N

The digestive system

concentrations of mercury were seen in the epithelium of ophageal glands and in the koilin of the gizzard Ten mercury appeared *in the intestinal mucosa and small* 'e in the intestinal contents The concentration increased injection but at the longer survival times no mercury pidly accumulated large amounts of mercury and showed ıg 29 30) There was a more marked localization of the portal veins Moderate amounts of mercury also the first days after injection In the exocrine pancreas light retention of mercury could be observed

Fig 27 Detail autoradiogram of a quail 10 days a jection of metr curic hydroxide retention of me epididymis and medullary cells

The urinary system

high concentration of mercury shortly after injecme of the birds the renal concentration of mercury *udy the uptake in the kidneys* was very prominent ock quails presented a more pronounced uptake ne hen quails (Fig 29) The major part of the in the proximal tubules Small amounts were *no mercury could be detected in the glomeruli* In the urine large amounts of mercury were



Fig 25 Autoradiogram (top) and corresponding section of the head of a hen quail 10 days after an i.v. injection of methoxyethyl mercuric hydroxide. A pronounced retention of mercury is seen in the anterior pituitary. The caudal lobe has a higher concentration than the cephalic lobe.

Fig. 26 Histochemical visualization of mercury (black grains see arrows) in a quail anterior pituitary 10 days after an i.v. injection of methoxyethyl mercuric hydroxide. Certain cells with a large pale nucleus and a large nucleolus, accumulate more mercury in their cytoplasm than other cells

Adrenal Kidney Epididymis Testis

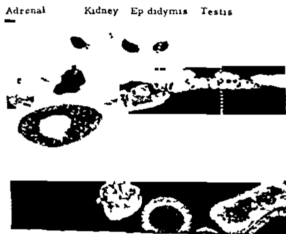


Fig. 27 Detail from an autoradiogram of a cock quail 10 days after i.v. injection of methoxyethyl mercuric hydroxide. Note the retention of mercury in the epididymis and in the adrenal medullary cells

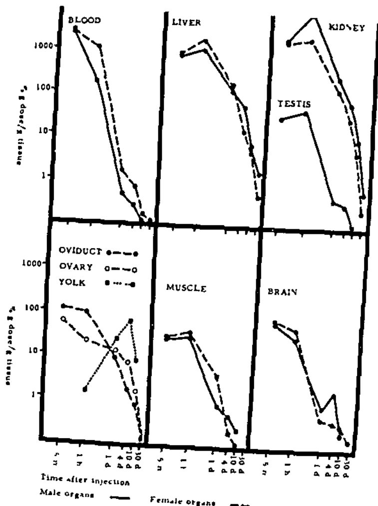


Fig 29 Concentration of mercury in some tissues of quails injected iv with a single dose of mercuric nitrate Each value represents the mean of three birds

The circulatory system

The blood retained relatively large amounts of mercury during the first days *after injection* The retention was more pronounced in the blood of the hen quails than in the blood of the cock quails (Fig 29) Up to four days *after injection* the myocardium showed an accumulation slightly higher than that of the skeletal *muscles* The bone marrow, the thymus and the spleen only accumulated small amounts of mercury

The nervous system

One day after injection there was a slight uptake of mercury *in* the brain and in the spinal cord The concentration slowly decreased with increasing *survival* time (Fig 29) The distribution within the brain was relatively even, but a higher uptake was seen in the choroid plexa, in the channel leading to the infundibular recess and in certain nuclear areas, e g the lateral nuclei of cerebellum and some nuclei in the midbrain High concentrations were also seen in the walls of many cerebral blood vessels The granular layer of the cerebellar cortex accumulated more mercury than the molecular layer Mercury also concentrated ...



Fig 30 Distribution of mercury (light areas) in a hen quail 4 days after an i v injection of mercuric nitrate Note the accumulation of mercury in kidney yolk and pituitary A ruptured follicle has spread mercury containing yolk in the abdominal cavity

cells in the sympathetic and spinal ganglia A high concentration was observed in ganglion cells situated in some cranial nerves probably N trigeminus and N facialis

The digestive system

After ingestion high concentrations of mercury were seen in the epithelium of the tongue in the oesophageal glands and in the koilin of the gizzard Ten minutes after injection mercury appeared in the intestinal mucosa and small amounts were also visible in the intestinal contents The concentration increased during the first day after injection but at the longer survival times no mercury was seen here The liver rapidly accumulated large amounts of mercury and showed a pronounced retention (Fig 29 30) There was a more marked localization of mercury in areas close to the portal veins Moderate amounts of mercury also appeared in the bile during the first days after injection In the exocrine pancreas and in the salivary glands a slight retention of mercury could be observed

The urinary system

The kidneys reached a very high concentration of mercury shortly after injec t on With increasing survival time of the birds the renal concentration of mercury eased but throughout the study the uptake in the kidneys was very prominent 30) The kidneys of the cock quails presented a more pronounced uptake the kidneys of the hen quails (Fig 29) The major part of the s localized in the proximal tubules Small amounts were whereas no mercury could be detected in the glomeruli 33) I he urine large amounts of mercury were

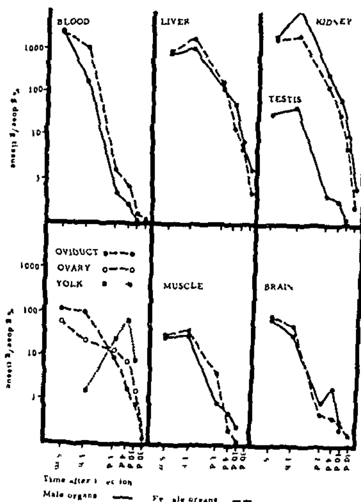


Fig 29 Concentration of mercury in some tissues of quails injected iv with a single dose of mercuric nitrate Each value represents the mean of three birds

The circulatory system

The blood retained relatively large amounts of mercury during the first days after injection The retention was more pronounced in the blood of the hen quails than in the blood of the cock quails (Fig 29) Up to four days after injection the myocardium showed an accumulation slightly higher than that of the skeletal muscles The bone marrow the thymus and the spleen only accumulated small amounts of mercury

The nervous system

One day after injection there was a slight uptake of mercury in the brain and in the spinal cord The concentration slowly decreased with increasing survival time (Fig 29) The distribution within the brain was relatively even but a higher uptake was seen in the choroid plexa in the channel leading to the infundibular recess and in certain nuclear areas e g the lateral nuclei of cerebellum and some nuclei in the midbrain High concentrations were also seen in the walls of many cerebral blood vessels The granular layer of the cerebellar cortex accumulated more mercury than the molecular layer Mercury also concentrated in ganglion

Day after injection	% of injected dose in eggs A	B	C
1	—	0 99	—
2	0 58	0 15	0 58
3	1 31	1 18	1 24
4	1 23	1 31	—
5	0 80	0 95	1 54
6	0 73	0 47	1 20
7	0 34	0 20	0 56
Excreted in eggs during 1 st week	4 99	5 25	5 12
2nd week	0 37	0 43	0 64
3rd week	0 07	0 04	0 06
Total	5 43	5 72	5 82

Table 4 Excretion of mercury in the eggs of three hen quails (A B, C) injected intravenously with a single dose of mercuric nitrate corresponding to 4 μg of mercury About 5 % of the dose was excreted this way mainly during the first week

Day after injection	% of injected dose in eggs A	B
1	—	1 18
2	4 12	7 28
3	12 68	15 76
4	7 02	10 42
5	7 48	7 68
6	6 28	3 00
7	3 13	3 27
Excreted in eggs during 1 st week	40 71	48 59
2nd week	6 63	3 98
3rd week	0 70	0 73
4th week	0 14	0 05
Total	48 18	53 35

Table 5 Excretion of mercury in the eggs of two hen quails (A B) injected intravenously with a single dose of mercuric nitrate corresponding to 50 μg of mercury The higher dosage resulted in an increased excretion as well totally as relatively About half the injected dose was recovered in the eggs during the first two weeks

The endocrine system

In the thyroid a moderate concentration of evenly distributed mercury was observed shortly after injection The parathyroid accumulated mercury slightly more than the thyroid The pancreatic islets did not show any marked uptake of mercury The adrenals presented a high uptake in the medullary cells (Fig 33)

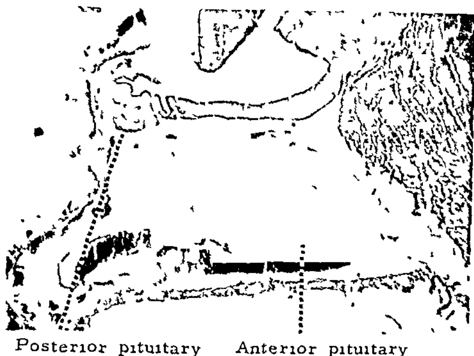


Fig 31 Microautoradiogram of the brain of a hen quail 10 days after an i v injection of *mercuric nitrate* An intense accumulation and retention of mercury (black areas) is presented by the anterior pituitary x 25



Fig 32 Microautoradi gram of a qua l kidney 1 day after an i v injecti n of mercuric nitrate The mercury (black grains) is ma nly l calized in the pr ximal tubules x (

Table 4 Excretion of mercury in the eggs of three hen quails (A B C) injected intra venously with a single dose of mercuric nitrate corresponding to 4 µg of mercury About 5 % of the dose was excreted thus way mainly during the first week

Day after injection	% of injected dose in eggs A	B	C
1	—	0 99	—
2	0 58	0 15	0 58
3	1 31	1 18	1 24
4	1 23	1 31	—
5	0 80	0 95	1 54
6	0 73	0 47	1 20
7	0 34	0 20	0 56
Excreted in eggs during 1 st week	4 99	5 25	5 12
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Table 5 Excretion of mercury in the eggs of two hen quails (A B) injected intravenously with a single dose of mercuric nitrate corresponding to 50 µg of mercury The higher dosage resulted in an increased excre tion as well totally as relatively About half the injected dose was recovered in the eggs du ring the first two weeks

Day after injection	% of injected dose in eggs A	B
1	—	1 18
2	4 12	7 28
3	12 68	15 76
4	7 02	10 42
5	7 48	7 68
6	6 28	3 00
7	3 13	3 27
Excreted in eggs during 1 st week	40 71	48 59
2nd week	6 63	3 98
3rd week	0 70	0 73
4th week	0 14	0 05
Total	48 18	53 35

The endocrine system

In the thyroid a moderate concentration of evenly distributed mercury was observed shortly after injection The parathyroid accumulated mercury *slightly* more than the thyroid The pancreatic islets did not show any marked uptake of mercury The adrenals presented a high uptake in the medullary cells (*Fig* 33)

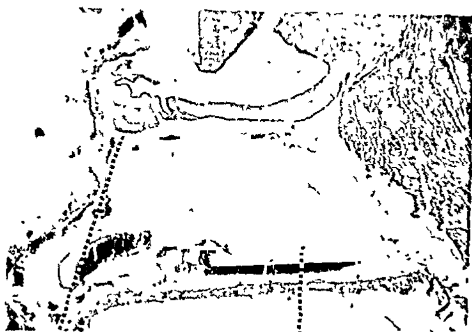


Fig 31 Microautoradiogram of the brain of a hen quail 10 days after an i v injection of mercuric nitrate *An intense* accumulation and retention of mercury (black areas) is presented by the anterior pituitary x 25



Fig 32 Microautoradiogram of a quail kidney 1 day after an i v injection of mercuric nitrate The mercury (black *grains*) *is* mainly localized in the proximal tubules x 6

Table 4 Excretion of mercury in the eggs of three hen quails (A B C) injected *intra* venously with a single dose of mercuric nitrate corresponding to 4 μg of mercury About 5 % of the dose was excreted this way *mainly during the first* week

Day after injection	% of injected dose in eggs A	B	C
1	—	0 99	—
2	0 58	0 15	0 58
3	1 31	1 18	1 24
4	1 23	1 31	—
5	0 80	0 95	1 54
6	0 73	0 47	1 20
7	0 34	0 20	0 56
Excreted in eggs during 1 st week	4 99	5 25	5 12
2nd week	0 37	0 43	0 64
3rd week	0 07	0 04	0 06
Total	5 43	5 72	5 82

Table 5 Excretion of mercury in the eggs of two hen quails (A B) injected intravenously with a single dose of mercuric *nitrate corresponding to 50 μg* of mercury The higher dosage resulted in an increased excretion as well totally as relatively About half the injected dose was recovered in the eggs during the first two weeks

Day after injection	% of injected dose in eggs A	B
1	—	1 18
2	4 12	7 28
3	12 68	15 76
4	7 02	10 42
5	7 48	7 68
6	6 28	3 00
7	3 13	3 27
Excreted in eggs during 1 st week	40 71	48 59
2nd week	6 63	3 98
3rd week	0 70	0 73
4th week	0 14	0 05
Total	48 18	53 35

The endocrine system

In the thyroid a moderate concentration of evenly distributed mercury was observed shortly after injection The parathyroid accumulated mercury slightly more than the thyroid The pancreatic islets did not show any marked uptake of mercury The adrenals presented a high uptake in the medullary cells (Fig 33)



Fig 33 Microautoradiogram of a quail adrenal 7 days after the bird was injected with mercuric nitrate The mercury (black grains) strongly concentrates in the medullary cells while less is seen in the interrenal cells x 64

During the first day after injection the anterior pituitary strongly concentrated mercury with a higher concentration in the caudal lobe than in the cephalic lobe The retention in the gland was marked (Fig 30, 31)

The reproductive system

The yolk of growing follicles intensely accumulated mercury which localized in the globules of both the white and the yellow yolk Some droplets contained considerably more than the average and this was especially true in a narrow zone indicating the time of injection (Fig 34)

The activity in the granular membrane and in the theca was relatively low No accumulation of mercury was observed in the yolk or shell membranes or in the shell

In the germinal discs a marked concentration of mercury was visible at the longer survival times Moderate concentrations of mercury appeared in the oviduct In the white of the eggs no mercury was seen on the autoradiograms but the impulse counting revealed small amounts during the first two weeks following injection (Fig 36) When the smaller dose was injected only about 5 % was excreted via the eggs during the first week but when the larger dose was given more than 10 % was excreted during this time (Table I 5) In the testes only a slight uptake was seen in the interstitial tissue and in the seminiferous epithelium The spermatogonia seemed to accumulate somewhat more mercury than



F g 34 Microautoradiogram of a follicle from a quail 1 day after injection of mercuric n tra c The stror g central concentration of me cury (black grains) represents the time of injection × 25



F x 35 Microautoradiogram of a quail testis and epididymis 10 days after injection of ercuric nitrate A strong uptake of mercury (black grains) is seen in the epithelium of w ene epididymal tubules × 25



Fig 33 Microautoradiogram of a quail adrenal 7 days after the bird was injected with mercuric nitrate The mercury (black grains) strongly concentrates in the medullary cells while less is seen in the interrenal cells x 64

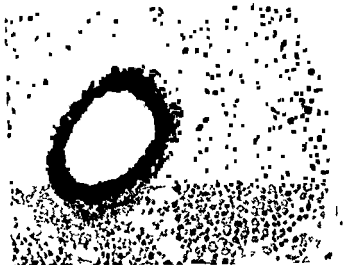
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The reproductive system

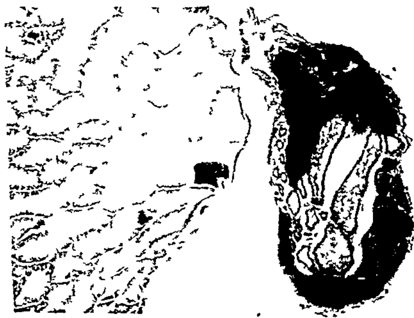
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F g 34 M croautorad ogram of a foll cle from a qua l 1 day after inject on of mercur c n tra e The strong cen ral concentrat on of me cury (black gra ns) represents the t me of n ect on x 25



F g 35 M croautorad ogram of a qua l test s and ep d dym s 10 days after inject on of m cur c n trate A strong uptake of mercury (black grains) s seen in the ep thel um of sem ep d dymal tubules × 25



Fig 33 Microautoradiogram of a quail adrenal 7 days after the bird was injected with mercuric nitrate The mercury (black grains) strongly concentrates in the medullary cells while less is seen in the interrenal cells x 64

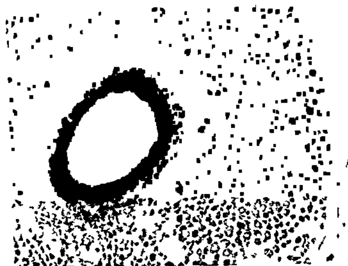
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The reproductive system

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F g 34 M croautorad ogram of a foll cle from a quail 1 day after injection of mercuric n ta e The strong central concentrat on of me cury (black gra ns) represents the time of nject on x 25

F g 5 Microautorad ogram of a quail test s and ep d dym s 10 days after njection of m cur c n rate A strong uptake of mercury (black gra ns) is seen in the epithelium of s me ep d dymal tubules × 25



Fig 33 Microautoradiogram of a quail adrenal 7 days after the bird was injected with mercuric nitrate The mercury (black grains) strongly concentrates in the medullary cells while less is seen in the interrenal cells x 64

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The activity in the granular membrane and in the theca was relatively low No accumulation of mercury was observed in the yolk or shell membranes or in the shell

In the germinal discs a marked concentration of mercury was visible at the longer survival times Moderate concentrations of mercury appeared in the oviduct In the white of the eggs no mercury was seen on the autoradiograms but the impulse counting revealed small amounts during the first two weeks following injection (Fig 36) When the smaller dose was injected only about 5 % was excreted via the eggs during the first week but when the larger dose was given more than 40 % was excreted during this time (Table 4 5) In the testes only a slight uptake was seen in the interstitial tissue and in the seminiferous epithelium The spermatogonia seemed to accumulate somewhat more mercury than

Discussion of the Distribution of Different Mercurials in the Quail (Chapter I—IV)

In the following are discussed the results of the previous four chapters Since very little information is available on the effects of mercurials on birds it has been necessary to make some comparisons with mammals and it should be remembered that species differences in anatomy and physiology sometimes make direct comparison difficult

In spite of a mercury accumulation in many tissues rather few organs are involved in the symptoms of mercury poisoning Whether an organic or inorganic mercurial the symptoms of acute poisoning mainly derive from the circulatory and renal systems and the symptoms of chronic poisoning from the nervous system The lack of symptoms from many organs which obviously accumulate mercury probably depends on the occurrence of protective factors such as binding to blood proteins cell membranes and unimportant but readily available sulphydryl groups, which protect more sensitive sites against damage (WEBB 1966) The importance of hereditary factors influencing the toxic response to a mercurial has also been shown in birds (MILLER *et al* 1959 b)

Much work has been made in vitro to study the effects of mercurials on tissue function Besides blocking most enzymes the mercurials react with many other proteins with the potential possibility of interfering with many physiological processes

In vitro experiments as a rule have been made using high concentrations of mercury on isolated cell structures and this together with the protective factors mentioned probably is the reason why many of the effects postulated from in vitro experiments never will manifest in the whole animal It must also be assumed that minor functional changes may not be discovered or attributed to poisoning with a mercurial

The distribution patterns of mercury in the quails which had received the various mercurials presented a great resemblance with the corresponding patterns observed in mammals Obviously methyl mercury held an exceptional position in comparison with the other mercurials It was distributed rather uniformly in the avian body most organs reaching a considerable concentration, whereas the other compounds resulted in a more differentiated uptake The remarkable affinity of methyl mercury to the oviduct and to the albumen was in contrast to the other compounds which resulted in a pronounced accumulation of mercury in the yolk

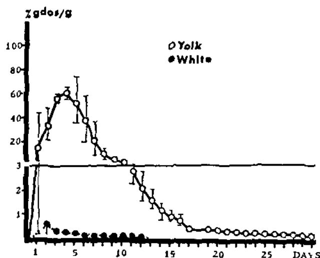


Fig 36 Concentration of mercury in yolk and egg white in eggs from quails injected with mercuric nitrate Each value represents two or three eggs

the other cells The epididymis intensely concentrated mercury in the epithelium of some tubules (Fig 35) Small amounts of mercury also appeared in the seminal fluid The epithelium of the ductus deferens also showed a relatively high uptake of mercury

Others

The initially high concentration of mercury in the lungs rapidly decreased and after four days only traces of mercury persisted A strong accumulation and retention appeared in parts of the nasal mucosa The skin showed a relatively large concentration of mercury A high concentration was seen in those parts of the feathers which were developed during the next few days after the injection (Fig 38) The skeletal muscles concentrated small amounts of mercury No mercury was observed in the bone but in some perichondral and periosteal connective tissue there was a very high uptake The subcutaneous fat depots showed a marked uptake of mercury during the first hours after injection but later only small amounts could be observed No mercury appeared in the uropygial gland or in the glycogen body In the eye the retina and the pecten showed low concentrations of mercury during the first days after injection

Incubated eggs

The chick accumulated mercury from the earliest stage of development The highest concentrations were seen in the internal yolk sac and in some portions of the intestines A moderate concentration was seen in the liver No or very little mercury was observed in the bile In the kidneys only moderate amounts appeared Low concentrations were seen in the central nervous system and in the skeletal muscles In perichondral bone relatively high concentrations of mercury were observed High concentrations also appeared in the cartilaginous epiphyses In spite of a pronounced accumulation in the feather papillae comparatively little mercury had concentrated in the juvenile plumage

adrenaline from isolated chromaffin granules of bovine adrenal medulla (DI IORIO 1957). The adrenal medullary cells originate from the sympathetic nervous system, which also accumulated mercury in its ganglia. The uptake in sympathetic and spinal ganglia may be responsible for certain symptoms of inorganic mercury poisoning. In rats conditioned reflexes are partially suppressed at a relatively low dose of mercuric chloride and at a higher dose there is a progressive loss of all the reflexes (GALOYAN 1957, GALOYAN & TURPAEV 1958, cit. WEBB 1966). In pink disease in children, caused i.a. by calomel and mercury ointments (WARKANY & HUBBARD 1953), loss of reflexes, muscular hypotonia and paresthesias were prominent symptoms. The nerve transmission in the cat superior cervical ganglion has also been demonstrated to be depressed by an organomercurial, p-mercuribenzoate (HALASZ *et al.* 1960). Degenerative changes in the sympathetic ganglia have also been described in human cases of mercury poisoning (GUILJAROWSKY & WINOKUROFF 1929).

The anterior pituitary presented an intense accumulation and retention of mercury. Methyl mercury seemed to localize uniformly in the gland which had a concentration equal to that in the brain. The other mercurials resulted in an uptake which sharply contrasted against the brain uptake and more mercury also appeared in the caudal lobe than in the cephalic lobe (for anatomy, see WINGSTRAND 1951). According to TIXIER VIDAL *et al.* (1968) the anterior pituitary of the quail contains seven types of cells, but at present it has not been possible to combine the microautoradiographic technique with the special staining methods necessary to identify these cells. The cytology of the quail pituitaries was similar to that described in laying fowls by PAYNE (1942) with numerous basophiles, comparatively less acidophiles and many chromophobes. After injection of inorganic mercury some cells seemed to accumulate more mercury than the average, and the histochemical demonstration of mercury after injection of methoxyethyl mercury also presented a higher concentration in the cytoplasm of certain cells staining acidophilically.

The higher concentration of mercury in the caudal lobe may be correlated to the alpha and gamma cells (TIXIER VIDAL *et al.* 1968) which occur exclusively in this part of the hypophysis. The functional significance of these cells is uncertain. The gamma cells probably are gonadotrophic and the alpha cells may be somatotrophic. PAYNE (1942) described two sorts of acidophils, A_1 and A_2, in the anterior pituitary of laying birds. Of these the A_1-cells occupy the caudal lobe and the A_2 cells the cephalic lobe. Both these cells have been attributed to the production of prolactin.

The avian pituitary elaborates all of the known hormones found in the mammalian gland and they exert the same or similar functions (STURKIE 1965). The pituitary hormones are concerned with a multiplicity of physiological processes and their nature of proteins or polypeptides suggests possibilities of a reaction with mercurials. They all contain sulphydryl and disulphide which are important for

Icterus has been described in chickens poisoned by mercurial fungicides (PALMER 1963) and BORG *et al* (1969) reported several cases of fatty degeneration. Concerning the accumulation in the intestinal mucosa, enteritis is a common symptom at acute mercury poisoning as well in birds as in mammals (TEJNING 1967 a, BORG *et al* 1969)

All the mercurials in the present investigation may cause nervous symptoms though with various character (BIDSTRUP 1964, HUNTER *et al* 1940, BORG *et al* 1969, TEJNING 1967 a, ZEYER 1952). After poisoning with methyl mercury the nervous symptoms dominate. Methyl mercury also presented a more pronounced uptake in the brain than the other compounds. The maximum value was reached several days after injection and the symptoms of poisoning with methyl mercury also manifest several days after its uptake (LUNDGREN & SWENSSON 1949, SWENSSON 1952). The autoradiograms of the other compounds showed a more differentiated distribution of mercury in the brain 4 days after injection than after 1 day, indicating a delayed uptake in certain parts. The accumulation in the choroid plexus and in the channel to the infundibular recess supports earlier suggestions that inorganic mercury enters the brain to a large extent via the cerebrospinal fluid (BERLIN & ULLBERG 1963 I)

The accumulation of mercury in the granular layer of the cerebellum which was observed after injection of methyl and inorganic mercury may be correlated to degenerative changes in this part of rat brains after poisoning of the rats with the same mercurials (HUNTER *et al* 1940, ENDERS & NOETZEL 1955). Pathological changes in the cerebellum of methyl mercury poisoned birds were reported by BORG *et al* (1969) and TAKEUCHI (1968). Inorganic mercury concentrated more strongly in some nuclear areas. The cerebellar nuclei e g presented a more pronounced uptake than surrounding tissues. Similar observations have also been made in mammals (BERLIN *et al* 1969, CASSANO *et al* 1969 NORDBERG & SERENIUS 1969). These nuclei are of importance for the movements of the neck (GROEBBELS 1929) and birds poisoned with methyl mercury often show symptoms which may be attributed to an injury at this site. This draws the attention to the question whether a part of the action of methyl mercury is due to an intracerebral release of inorganic mercury. Methyl mercury penetrates the brain intact (GAGE 1964) and the latency period before the neurological symptoms manifest may well be correlated to a small intracerebral release of inorganic mercury. The delayed uptake in the brain and a different reactivity between methyl mercury and various binding sites are other factors which must also be considered when discussing this latency of the symptoms

Mercury concentrated and was retained in the avian adrenals especially in the medullary cells. Degenerative changes have been observed in mammalian adrenals after mercury poisoning and the medulla has been shown to release its contents of catecholamines (GRANZOW 1926 HESSE 1925). Some organo mercurials have also been demonstrated to release large amounts of adrenaline and nor

adrenaline from isolated chromaffin granules of bovine adrenal medulla (DIORIO 1957) The adrenal medullary cells originate from the sympathetic nervous system which also accumulated mercury in its ganglia The uptake in sympathetic and spinal ganglia may be responsible for certain symptoms of in organic mercury poisoning In rats conditioned reflexes are partially suppressed at a relatively low dose of mercuric chloride and at a higher dose there is a progressive loss of all the reflexes (GALOYAN 1957, GALOYAN & TURPAEV 1958, cit WEBB 1966) In pink disease in children, caused i a by calomel and mercury ointments (WARKANY & HUBBARD 1953), loss of reflexes, muscular hypotonia and parestesias were *prominent symptoms* The nerve transmission in the cat superior cervical ganglion has also been demonstrated to be depressed by an organomercurial, p mercuribenzoate (HALASZ *et al* 1960) Degenerative changes in the sympathetic ganglia have also been described in human cases of mercury poisoning (GUILJAROWSKY & WINOKUROFF 1929)

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their biological activity (van DYKE *et al* 1943, LI & EVANS 1938) Binding to other components, e g enzymes involved in the hormonal synthesis must also be considered, and the pituitary sinusoids, like those in the liver and in the spleen, are lined with phagocytes, and an uptake of mercury here would contribute to the accumulation The mouse pituitary does not accumulate mercury (BERLIN & ULLBERG 1963 I, II, III) In man, however, high concentrations of mercury have been demonstrated in the pituitary (STOCK 1940, AHLMARK 1948), and it is interesting to note that the A_1 cells of Payne are considered very similar to the alpha cells of mammals The question why the quail pituitary so intensely accumulated mercury must for the present remain unanswered In view of the reproductive behaviour of this avian species, a strong production of hormones may be involved

Another question is whether the uptake of mercury in the anterior pituitary affects the hormones produced there and their physiological response At present very little information supporting such effects is available The increased frequency of "floor eggs observed by BORG *et al* (1965) and TEJNING (1967 a), may indicate a decreased activity of prolactin which regulates the broody behaviour of birds In this connection the higher concentration of mercury in the caudal lobe of the pituitary should be remembered, because NAKAJO & TANAKA (1956) demonstrated the caudal lobe of broody hens to have a higher potency of prolactin than the cephalic lobe The decreased laying frequency observed by TEJNING (1967 a) and by HELMINEN *et al* (1966) may also be attributed to a hypophyseal effect of mercury Ovulation is induced by the release of luteinizing hormone (LH) (MARSHALL 1960), probably produced in the gamma cells of the caudal lobe (HERLANT *et al* 1960) LH is also of importance for the production of estrogen which stimulates the deposition of calcium in the medullary bone (STURKIE 1954) The increased frequency of shell less eggs observed by TEJNING (1967 a) may be attributed to a calcium deficiency

If we focus the interest to the avian reproductive apparatus it is obvious that this was attacked by the mercurials at almost every point The accumulation of mercury in the pituitary may suggest an influence on the gonadotrophic hormones Methyl mercury selectively accumulated in the oviduct and in the albumen while the other compounds resulted in an uptake of mercury in the yolk and in the germinal discs In the testes there was a moderate accumulation of methyl mercury while inorganic mercury and methoxyethyl mercury mainly localized in the epididymis

The amount of mercurial injected was of great importance for the accumulation of mercury in the eggs When the dose was increased relatively more mercury appeared in the eggs This also explained why more mercury was seen in the eggs on the autoradiograms than could be expected from the quantitative values which were obtained from birds injected with a lower dose of the mercurials The egg evidently acted as a safety valve excreting the excess of mercury not excreted

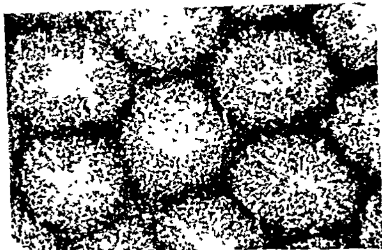


Fig 37 Microautoradiogram of a mouse testis 10 days after an i v injection of mercuric nitrate A very pronounced accumulation of mercury (black grains) is seen in the interstitium whereas less can be observed in the seminiferous epithelium (BACKSTRÖM unpublished)

by other organs This was further illustrated by the sex differences after injection of methyl mercury and it seems reasonable to assume the laying of eggs to be a most important maybe life saving factor in the avian excretion of methyl mercury The highest values of mercury in the eggs were also seen after injection of methyl mercury whereas injection of the other compounds which evidently found other ways of excretion resulted in lower values During the late 1950 s a severe reduction of the pheasant populations occurred in southern Sweden as a result of unsuccessful reproduction attributed to methyl mercury poisoning (BORG *et al* 1969) Some observations also indicated the presence of more hen pheasants than of cock pheasants

In growing follicles mercury concentrated as well in yellow as in white yolk The higher concentrations observed in the thin layers of white yolk alternating with the broader layers of yellow yolk probably were the result of the diurnal variations in the formation of yolk The narrow layers of white yolk, formed more slowly than the layers of yellow yolk, obviously permit more mercury to concentrate in spite of the fact that the white yolk contains considerably less protein and lipids than the yellow yolk (ROMANOFF & ROMANOFF 1949) In some eggs a higher concentration of mercury also appeared in layers of yellow yolk developed later after injection of the mercurial than layers with less mercury This must be attributed to a temporary retardation in the formation of the yolk. The higher concentrations of mercury seen in many yellow yolk droplets, surrounded by droplets with less activity may also depend on a different rate of formation



Fig 38 Distribution of mercury in quail feathers A feather and B corresponding auto radiogram from a bird which was given a single i v injection of mercuric nitrate C auto radiogram of a feather from a bird given a single i v injection of methyl mercuric nitrate Feather A was a few mm long when the injection was given whereas feather C had not yet appeared The feathers were obtained from birds 30 days after injection of the mer curials The methyl mercury is continuously deposited in the growing feather Injection of the inorganic mercurial results in a gradually decreasing uptake

literature (MURAKAMI *et al* 1955), and since many teratogenic agents exert their effect by denying certain enzymes to the embryo the inhibitory effects of mercurials on enzymes may suggest a similar action

Administration of the mercurials resulted in a strong accumulation of mercury in the plumage A continuous uptake was seen in developing feathers after injec tion of methyl mercury, while the other compounds presented a gradually dec reasing concentration with a high uptake in parts developed during the days next to injection (Fig 38) In a cock fed methyl mercury treated seed, TEJNING (1967 a) recovered 95 % of the deposited mercury in the plumage No observa tions have been found on an impaired quality of the feathers of mercury poisoned birds, but it has been demonstrated that mercury weakens keratin fibres so that they break under less tension (HOARE & SPEAKMAN 1963)

The feather autoradiograms have not demonstrated the diurnal variations in the growth of the feathers and the development of the growth bars, as shown by LÜDICKE (1959) after injection of radioactive sulphur in various birds Great species differences in the appearance of these growth bars, however, occur (LÜDICKE 1961)

In some feathers mercury was observed at low concentrations in parts of the vane developed before the mercurial was given. The source of this mercury is uncertain The uropygial gland showed no uptake of mercury, but the possibility still must be considered that some mercury was transported to the plumage via the secretion of this gland The possibility of an external contamination, eventually aerial, from the droppings of the birds also exists

Much work with proteins and enzymes have demonstrated the sulphydryl groups as the primary site of mercurial binding, but as pointed out by HUGHES (1950, 1957), mercurials will also complex with other groups once the sulphydryl groups are saturated Histochemical methods have visualized the distribution of sulphydryl and disulphide groups in mammals, and if we assume a similar distribution in birds, there is a great resemblance to the distribution of mercury BARRNETT (1953) and BARRNETT & SELIGMAN (1954) studied the distribution of sulphydryl and disulphide groups in the rat, and many tissues which accumulate mercury obviously are rich in these groups The different distribution pictures of the various mercurials, however, indicate that other factors than a simple binding to sulphur are involved Such factors may be lipid solubility, stereochemistry and membrane passage

CHAPTER FIVE

Comparison of the Distribution of ^{203}Hg- and ^{14}C-Labelled Methyl and Phenyl Mercury in the Quail

The symptoms of poisoning with methyl mercury very markedly differ from those of poisoning with phenyl mercury (SWENSSON & ULFVARSON 1963) This difference in toxicity has been correlated to a different metabolism and body distribution of these compounds The alkyl mercury radical is considered very stable in the body, but there are indications of a small degradation to inorganic mercury (MILLER *et al* 1961, GAGE & SWAN 1961, GAGE 1964) Phenyl mercury has been found to decompose quite rapidly to inorganic mercury (MILLER *et al* 1960, GAGE & SWAN 1961) and its administration also gives a distribution picture resembling that of inorganic mercury (BERLIN & ULLBERG 1963 I, II)

The aim with the present investigation was to study, by means of whole body autoradiography, the stability of the mercury carbon bond of the mentioned organo mercurials in quails This was made by comparing a series of animals intravenously administered the compounds labelled with the radioactive isotope ^{203}Hg with another series of animals administered the same compounds labelled with the radioactive isotope ^{14}C The distribution pictures showed if the two isotopes had followed each other or separated

MATERIALS AND METHODS

^{14}C labelled methyl mercuric hydroxide and phenyl mercuric nitrate were synthesized by fil kand O Borgå, Institute of Analytical Chemistry University of Stockholm, Sweden The labelled compounds were purified from radioactive impurities according to ÖSTLUND (1969) The specific activity was for the methyl mercury 150 μCi/mg mercury, and for the phenyl mercury 80 μCi/mg mercury The ^{14}C methyl compound, dissolved in water and the ^{14}C phenyl compound dissolved in DMSO ethanol acetone (75 20 5) were injected intravenously in the animals The dose calculated as mercury, was 1 mg/kg body weight The distribution pictures of the ^{203}Hg labelled compounds were obtained from the previous avian experiments The survival times of the birds were 10 minutes 1 hour 4 hours 1 day 4 days and 10 days Whole body autoradiography was performed according to ULLBERG (1954 1958) as previously described

RESULTS AND CONCLUSIONS

1 Stability of methyl mercury

The distribution pictures of the two isotopes were *almost identical at all the survival times studied*, which speaks in favour of a great stability of methyl mercury (Fig 39, 40) No significant signs indicating a break down of the methyl mercury radical could be observed In both cases the radioactivity was quite evenly distributed in the body except concerning the oviduct, the albumen, the plumage and the bill, which presented an intense uptake

The renal concentration of ^{14}C was lower than that in the liver after all the survival times studied After intravenous injection of ^{203}Hg labelled methyl mercury, the isotope concentration in the kidneys was somewhat higher than that in the liver However, the ^{203}Hg labelled methyl mercury contained a small impurity of inorganic mercury which probably accounts for the difference. MILLER *et al* (1961) found a higher concentration of mercury in the liver than in the kidneys of chicken given ethyl mercury perorally and intramuscularly, whereas SWENSSON & ULFVARSON (1968 a) observed a higher concentration of mercury in the kidneys than in the liver of cocks given methyl mercury intravenously The latter authors did not mention the radiochemical purity, and since it is known that many organo mercurials may decompose slowly in aqueous solutions (MUDGE & WEINER 1958), an impurity of inorganic mercury may explain the higher renal uptake A different dosage may, however, also influence the result. A small decomposition of methyl mercury to inorganic mercury, especially during the first day after injection, would, however, contribute to a higher uptake of ^{203}Hg in the kidneys and in the yolk, and cannot be excluded That this may occur is supported by MILLER *et al* (1961) who reported a small decomposition of ethyl mercury in chicks Autoradiography has also indicated an initial decomposition of methyl mercury in mice (BACKSTROM to be published) This, however, seemed to be quite small, possibly due to an inhibition of the participating enzymes by the inorganic mercury released

2 Stability of phenyl mercury

The distribution patterns of the two isotopes rapidly grew different, indicating a break of the carbon mercury bond Initially they were quite similar, but 1 hour after injection certain differences appeared In both cases the highest isotope concentration was seen in the kidneys but, in comparison with other organs, the lungs showed a much higher concentration of ^{14}C than of ^{203}Hg More ^{14}C than ^{203}Hg was also observed in the intestinal contents and in the koilin lining of the gizzard

One day after injection the differences were more pronounced ^{203}Hg strongly concentrated in the kidneys, in accumulating yolk and in the liver (Fig 12) The blood concentration of ^{203}Hg was about equal to that in the skeletal muscles and

CHAPTER FIVE

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Fig 41, where the diminished uptake in the periphery of the egg white suggests that the decomposition is completed one day after the injection

Irrespective of the radioactive label, the lungs showed a higher isotope activity than the blood as early as 10 minutes after injection, suggesting a special pulmonary binding site The retention of ^{14}C in the lungs may also support this idea, and the question arises whether the lungs play some active role in the metabolism of phenyl mercury in quails Similar experiments with "double labelled phenyl mercury in mice, have not demonstrated any marked retention of ^{14}C in the mouse lung though many other signs pointed at a rapid decomposition of the compound (BACKSTRÖM to be published)

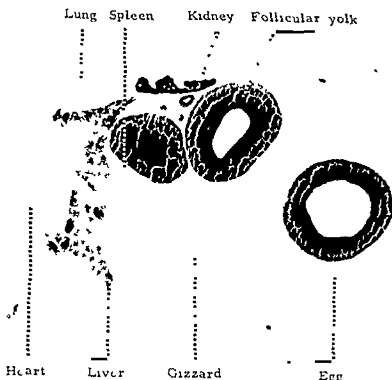


Fig 42 Autoradiogram of a hen quail 4 days after an i v injection of ^{203}Hg labelled phenyl mercury The mercury isotope has concentrated in the kidney and in the egg yolk

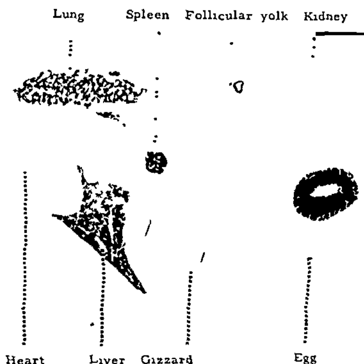


Fig 43 Autoradiogram of a hen quail 4 days after an i v injection of ^{14}C labelled phenyl mercury The carbon isotope is retained in the lung and in the spleen No ^{14}C is observed in the follicular yolks The uptake in the yolk of the egg ready to be laid represents the time when the mercurial was intact

(Packard) The radioactivity in the blood corpuscles was then calculated as was the ratio of mercury between blood corpuscles and blood plasma The percentage of non protein bound activity was checked by precipitation of the plasma protein with ethanol to a final concentration of 40 %

2 Electrophoresis of blood plasma proteins

Zone electrophoresis of the plasma samples obtained above and from two cock quails injected with mercuric nitrate (0 25 mg/kg) was carried out on cellulose acetate strips (Sepraphore III, Gelman Instrument Company, USA) The buffer used was Gelman High Resolution Buffer (pH 8 8, μ = 0 05) and 10 μl of each plasma sample was applied on each strip The electrophoresis was run for 60—90 minutes with 1 5 mamp per strip whereafter the proteins were fixed and stained in 0 5 % Ponceau S in a 5 % aqueous solution of trichloracetic acid Some strips were also stained for lipoproteins according to the ozone Schiff technique (KOHN 1961) Electropherograms were also obtained on Sartorius cellulose acetate strips (Sartorius Membranfilter GmbH, Gottingen, West Germany) immersed in a barbitone buffer (pH 8 6, μ = 0 05) After electrophoresis the strips were autoradiographed on X ray film (Structurix, Gevaert) The electropherograms representing the shortest survival times were also scanned in a radiochromatogram scanner (Packard)

3 Electrophoresis of egg white and yolk proteins

Zone electrophoresis was performed, as described above, with egg white and yolk of some eggs from the hens used in the autoradiographic experiments with mercuric nitrate and methyl mercuric nitrate The electrophoresis was run with homogenized yolk, egg white and a mixture of homogenized egg white and yolk The yolk samples were withdrawn by a syringe in order to avoid contamination with albumen The samples were slightly diluted with buffer Dialysis against the buffer was made with a few samples but was considered unnecessary and therefore not further applied

Samples were also electrophoresed after incubation for 5 and 30 minutes at room temperature with ^{203}Hg labelled mercuric nitrate and methyl mercuric nitrate (0 02 mg mercury per gram sample) The electropherograms were autoradiographed and stained as described above

RESULTS

Regardless of the mercurial injected, all, or almost all, of the mercury in the blood plasmas was bound to the proteins None or less than 1 % of the activity remained in solution when the proteins were precipitated by alcohol

The distribution of mercury between the blood corpuscle and plasma fractions markedly differed depending on the type of mercurial injected At all times

CHAPTER SIX

Distribution of Mercury in Blood and Egg Proteins of the Quail

In the previous chapters there were indications that the organic mercurials were retained in the circulating blood for a longer time than the inorganic mercury. This was especially true after injection of methyl mercury in the cock quails which also showed a much stronger retention than did the hens. Concerning the other mercurials, including mercuric nitrate, the hens instead retained more mercury in the blood than the cocks.

In chicken administered ethyl mercury MILLER *et al.* (1961) observed about 90 % of the mercury in the blood to be bound to the blood corpuscles and in domestic hens TEJNING (1967) found a very high affinity of methyl mercury to the blood corpuscles whereas little was bound in the plasma. That organo-mercurials are bound to the blood corpuscles to a larger extent than inorganic mercury has also been observed in mammals by several investigators, i.e. SWENSSON *et al.* (1959), BERLIN (1963), GAGE (1964) and YOSHINO *et al.* (1966), and a longer persistence of organo-mercurials than of inorganic mercury in the blood has also been demonstrated (BERLIN & ULLBERG 1963 I, II, III).

In the present investigation the distribution of mercury in the main blood fractions of quails injected with various mercurials has been studied by scintillation counting and zone electrophoresis combined with autoradiography.

Electrophoresis has also been used to study the distribution of mercury in the yolk and egg white proteins. In birds certain changes appear in the blood proteins at the onset of laying (LUSH 1963) and since many attempts have been made to correlate between the plasma and egg proteins, it was considered interesting to compare the blood and egg distribution of mercury.

MATERIALS AND METHODS

1. The ratio of mercury between blood corpuscles and blood plasma

Four series of laying quails, each series consisting of 4 birds, were injected intravenously with the same ^{203}Hg labelled mercurials as were used in the previous autoradiographic experiments. The dose calculated as mercury was 0.25 mg/kg body weight. Ten minutes, 1 hour, 1 day and 10 days after injection samples of blood (0.2 ml) were drawn into a heparinized syringe from a wing vein. The hematocrit values were determined and the radioactivity in weighed samples of whole blood and blood plasma was measured in a well-type scintillation crystal

(Packard) The radioactivity in the blood corpuscles was then calculated as was the ratio of mercury between blood corpuscles and blood plasma The percentage of non protein bound activity was checked by precipitation of the plasma protein with ethanol to a final concentration of 40 %

2 Electrophoresis of blood plasma proteins

Zone electrophoresis of the plasma samples obtained above and from two cock quails injected with mercuric nitrate (0 25 mg/kg) was carried out on cellulose acetate strips (Sepraphore III, Gelman Instrument Company, USA) The buffer used was Gelman High Resolution Buffer (pH 8 8, μ = 0.05) and 10 μl of each plasma sample was applied on each strip The electrophoresis was run for 60—90 minutes with 1 5 mamp per strip whereafter the proteins were fixed and stained in 0 5 % Ponceau S in a 5 % aqueous solution of trichloracetic acid Some strips were also stained for lipoproteins according to the ozone Schiff technique (KOHN 1961) Electropherograms were also obtained on Sartorius cellulose acetate strips (Sartorius Membranfilter GmbH Gottingen, West Germany) immersed in a barbitone buffer (pH 8 6, μ — 0 05) After electrophoresis the strips were autoradiographed on X ray film (Structurix, Gevaert) The electropherograms representing the shortest survival times were also scanned in a radiochromatogram scanner (Packard)

3 Electrophoresis of egg white and yolk proteins

Zone electrophoresis was performed, as described above, with egg white and yolk of some eggs from the hens used in the autoradiographic experiments with mercuric nitrate and methyl mercuric nitrate The electrophoresis was run with homogenized yolk, egg white and a mixture of homogenized egg white and yolk The yolk samples were withdrawn by a syringe in order to avoid contamination with albumen. The samples were slightly diluted with buffer Dialysis against the buffer was made with a few samples but was considered unnecessary and therefore not further applied

Samples were also electrophoresed after incubation for 5 and 30 minutes at room temperature with ^{203}Hg labelled mercuric nitrate and methyl mercuric nitrate (0 02 mg mercury per gram sample) The electropherograms were autoradiographed and stained as described above

RESULTS

Regardless of the mercurial injected all or almost all, of the mercury in the blood plasmas was bound to the proteins None or less than 1 % of the activity remained in solution when the proteins were precipitated by alcohol.

The distribution of mercury between the blood corpuscle and plasma fractions markedly differed depending on the type of mercurial injected At all times

Type of mercurial	Time after injection			
	10 minutes	1 hour	1 day	10 days
Inorganic mercury	0 8 (0 4—1 3)	0 6 (0 4—0 9)	0 4 (0 2—0 7)	0 9 (0 7—1 2)
Methyl mercury	12 7 (9 6—15 4)	32 5 (30 0—34 9)	28 2 (20 2—31 6)	28 7 (25 4—32 6)
Phenyl mercury	16 5 (12 4—21 2)	26 2 (19 7—30 5)	1 6 (1 2—1 8)	0 5 (0 4—0 7)
Methoxy ethyl mercury	5 3 (3 9—7 9)	4 4 (4 1—4 7)	1 1 (0 9—1 5)	0 3 (0 2—0 5)

Table 6 Ratio of mercury between blood corpuscles and blood plasma in quails injected i v with various mercurials Each value represents 4 birds Inorganic mercury is somewhat more bound to the plasma proteins than to the blood corpuscles (a low ratio) while methyl mercury mainly binds to the blood corpuscles (a high ratio) Phenyl and methoxyethyl mercury first bound to the blood corpuscles show a redistribution of mercury, probably due to a decomposition to inorganic mercury

studied, injection of inorganic mercury resulted in a low ratio, whereas injection of methyl mercury resulted in a high ratio (Table 6) When phenyl or methoxy ethyl mercury was injected the ratio was initially high, but 1 and 10 days later it had markedly decreased

The electropherograms of the blood plasmas on the Sepraphore strips showed considerable variations in the protein pattern as well between different hens as between samples taken at different times from the same hen The plasmas from the cocks also markedly differed from those of the hens The nomenclature of the avian plasma proteins is somewhat obscure, but the fractions resemble in electrophoretic mobility those of the human (BUEKER 1960) The patterns obtained were also very similar to those observed in turkey and fowl (COMMON *et al* 1953) with five main fractions corresponding to albumin, alpha$_1$ globulin alpha$_2$ globulin, beta globulin and gamma globulin of the human plasma

In the plasmas of the hen quails the α_1 globulin was often markedly reduced In addition these plasmas displayed two small prealbumin bands and a slowly migrating fraction strongly positive for the ozone Schiff staining This lipoprotein fraction seemed comparable with the lipophosphoprotein complex (PLP) described by McINDOE (1959) and with the presumptive phosphoprotein (PP) and presumptive lipovitellin (PLV) described by COMMON *et al* (1953) and McKINLEY *et al* (1953)

The Sartorius electrophoresis strips separated a prealbumin, albumin two alpha globulins and one band with beta and gamma globulins

The following observations of the mercury distribution in the blood plasma were the most conspicuous

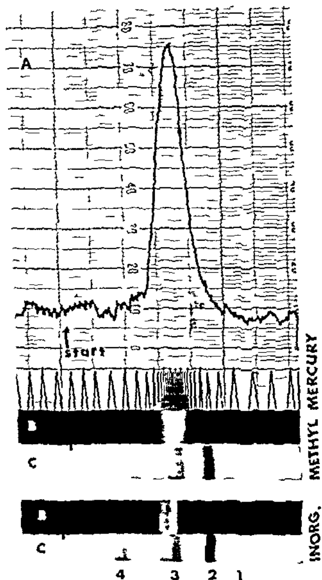


Fig. 44 Distribution of mercury in blood plasma proteins of laying hen quails 1 hour after an i.v. injection of methyl mercury (top) and *inorganic* mercury (bottom). Electropherogram (Sartorius). A scanned. B autoradographed. C ...

... *es in the α globulins.* ... ury. In

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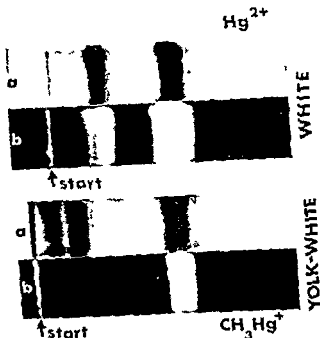


Fig 46 (top) Egg white incubated for 5 min with mercuric nitrate The mercury is localized in the ovalbumin and in the ovomucoid-conalbumin regions
(bottom) Yolk + egg white incubated for 5 min with methyl mercuric nitrate The mercury is localized in the ovalbumins only Electropherogram (Gelman), a stained with Ponceau S b autoradiographed

zone with a lower mobility containing the conalbumins, ovomucoid and ovoglobulins (LONGSWORTH *et al* 1940)

With the exception of a faint uptake in the ovalbumins, no mercury was seen in the egg white proteins of the quails given mercuric nitrate On the other hand, egg white incubated with mercuric nitrate showed a pronounced accumulation in the ovalbumin as well as in the ovomucoid conalbumin bands (Fig 46)

When the birds were injected with methyl mercury only the ovalbumins accumulated mercury (Fig 47) The localization was the same when the mercurial was added in vitro

The electropherograms of the yolk samples displayed two slow moving fractions strongly positive for lipids and two faster moving bands, less rich in proteins and not positive for lipids The lipoprotein bands appear identical with the presumptive phosphoprotein zone of McKINLEY *et al* (1953), and probably contain lipovitellenin and lipovitellin (FEVOLD 1951) The two fast moving fractions may be correlated to the Y and X bands as described by McKINLEY *et al* (1953)



Fig 45 Distribution of mercury in blood plasma proteins of a laying hen quail (top) and a cock (bottom) 1 hour after an *i v* injection of mercuric nitrate Electropherogram (Gelman) a stained with Ponceau S, b autoradiographed, c stained for lipids
1 albumin, *2* and *3* region of α globulins, *4* region of β and γ globulins, 5 lipoprotein In the cock plasma the mercury migrates in two bands with a slower mobility than the mercury binding bands in the hen plasma

In no case mercury migrated in the prealbumin or albumin fractions After injection of inorganic mercury in laying hens the mercury, at all times studied was localized in the α globulin region (Fig 44) The major part seemed to migrate in the α_1 globulin band In the cock plasmas the mercury was instead mainly localized in the α_2 and in the β-globulin bands (Fig 45) The plasma bound part of the methyl mercury migrated similarly as the inorganic mercury (Fig 44)

Injection of phenyl mercury also resulted in an uptake of mercury in the α globulins of the hens Ten minutes and 1 hour after injection the β and γ globulin bands, however, had a higher uptake than the α globulin bands, but after 1 day mercury was only seen in the latter Administration of methoxyethyl mercury presented a similar migration of mercury

The electropherograms of the egg white samples displayed four fast moving bands, close to each other, which were considered to be ovalbumins, and a broader

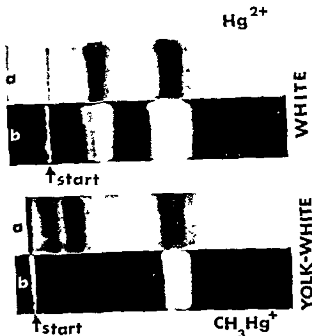


Fig 46 (top) Egg white incubated for 5 min with mercuric nitrate The mercury is localized in the ovalbumin and in the ovomucoid conalbumin regions
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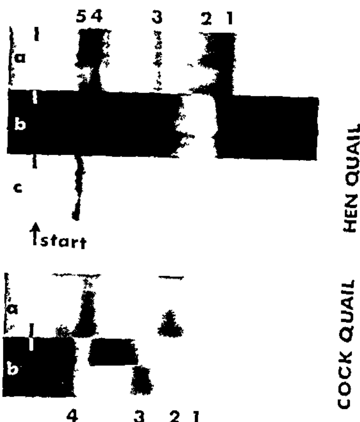


Fig 45 Distribution of *mercury in blood* plasma proteins of a laying hen quail (top) and a cock (bottom) 1 hour after *an i v injection* of mercuric nitrate Electropherogram (Gel man) a stained with Ponceau S b *autoradiographed* c stained for lipids
1 albumin 2 and 3 *region of* α globulins 4 region of β and γ globulins 5 lipoprotein In the cock plasma the mercury *migrates* in two bands with a slower mobility than the mercury binding bands in the hen plasma

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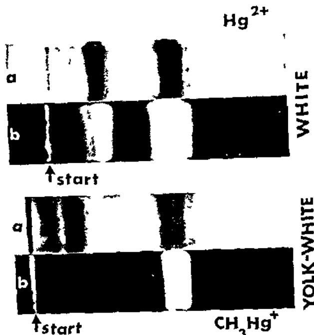


Fig 46 (top) Egg white incubated for 5 min with mercuric nitrate The mercury is localized in the ovalbumin and in the ovomucoid conalbumin regions
(bottom) Yolk + egg white incubated for 5 min with methyl mercuric nitrate The mercury is localized in the ovalbumins only Electropherogram (Gelman), a stained with Ponceau S b autoradiographed

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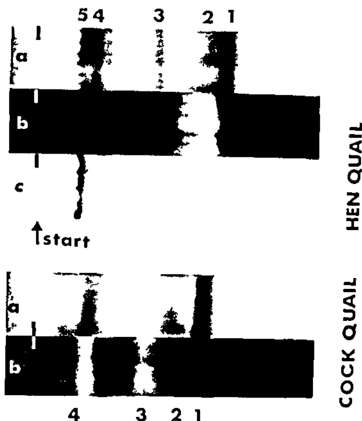


Fig 45 Distribution of mercury in blood plasma proteins of a laying hen quail (top) and a cock (bottom) 1 hour after an i v injection of mercuric nitrate Electropherogram (Gelman) a stained with Ponceau S b autoradiographed c stained for lipids
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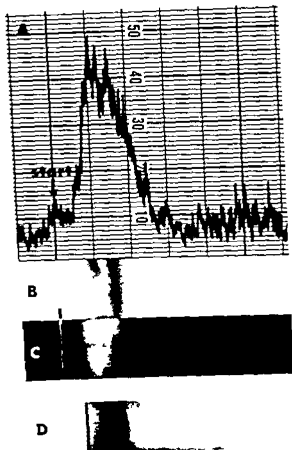


Fig 48 Distribution of mercury in yolk proteins from a quail injected with mercuric nitrate The mercury migrates in two bands positive for lipid staining probably lipovitellenin and lipovitellin Electropherogram (Gelman) A scanned B stained for lipoproteins C autoradiographed D stained with Ponceau S

DISCUSSION

A binding of mercury to the serum albumins was never seen in the quails This is interesting in view of the great capacity of albumins to bind many physiological as well as unphysiological substances Albumin has a much stronger tendency to bind such substances than has the other plasma proteins In mouse and fish plasma mercury also readily combines with the albumins (ROTHSTEIN & HAYES 1960 BACKSTRÖM unpublished) The reason for this marked species difference is unknown at present HUGHES (1947) has characterized mercaptalbumin, a fraction of human serum albumin containing one reactive sulphydryl group per molecule as the binding site of mercury in human blood serum It seems reasonable to assume the absence of this readily available sulphydryl group in the quail albumins

The ratio of mercury concentration between blood corpuscles and plasma was low after injection of mercuric nitrate which indicated that more mercury was

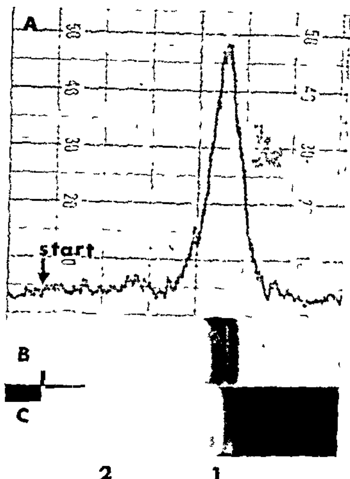


Fig 47 Distribution of mercury in egg white proteins from a quail injected i v with methyl *mercuric nitrate* Only the ovalbumins retain mercury Electropherogram (Gelman) A scanned, B stained with Ponceau S C autoradiographed
1 ovalbumin, 2 ovomucoid conalbumin region

In yolk from hens injected with mercuric nitrate, the mercury migrated in the lipoprotein fractions (Fig 48) Incubating of yolk with mercuric nitrate gave the same result

The yolk samples incubated with methyl mercury and those from the hens injected with methyl mercury did not contain migrating amounts of mercury

The electropherograms of the yolk-albumen mixtures presented combinations of the results above In eggs administered inorganic mercury in vivo, the mercury, as before, only localized in the lipoproteins, whereas eggs added the mercurial in vitro presented an uptake in both yolk and white proteins

The electrophoresed yolk albumen mixtures of the eggs from the hens given methyl mercury only had an uptake of mercury in the ovalbumin bands Incubation of a yolk-albumen mixture with methyl mercury also resulted in a binding only to the ovalbumins (Fig 46)

structure of a micelle with the separate protein molecules held together by lipid (MARTIN *et al* 1959)

In vivo inorganic mercury bound only to the lipoproteins of the yolk but in vitro it also combined with the ovalbumin and ovomucoid-conalbumin fractions The methyl mercury, on the other hand bound as well in vivo as in vitro only to the ovalbumins Ovalbumin is the classical example of a protein which contains, in its native state, so-called masked sulphydryl groups, and in their native state ovalbumin, conalbumin and ovomucoid react sluggish or not at all with various sulphydryl reagents (ANSON 1941, FEVOLD 1951) After denaturation the sulphydryl groups are unmasked and readily react with these reagents It has, however, been demonstrated by HUGHES (1950) that methyl mercuric nitrate rapidly reacts with native ovalbumin, which is in agreement with the present observation. Methyl mercuric nitrate did not react with the ovomucoid conalbumin fraction as did mercuric nitrate, which also combined in vitro with the ovalbumins Ovalbumin is rich in methionine and cysteine (KASSEL & BRAND 1938) and a binding of methyl mercury to the sulphur of these amino accids seems convincing Stereochemical arrangements may be responsible for the binding of the various mercurials to the various proteins Of importance for the in vivo uptake of the mercurials in egg white or yolk respectively, may also be the fact that the yolk proteins are synthesized in the liver and are then transported in the blood to the ovary The white proteins on the other hand, are synthesized in the oviduct and are consequently not exposed to the mercurials in the blood Inorganic mercury which in vitro combines with the egg white as well as with the yolk proteins in vivo first meets the yolk proteins in the blood and is bound to these A small binding to the white proteins in the oviduct seems reasonable and a slight uptake of inorganic mercury was also observed in the ovalbumin fractions In the autoradiograms a moderate accumulation was also observed in the oviduct. The question arises why the methyl mercury a part of which actually migrated with the α globulins is not incorporated in the yolk proteins The uptake of methyl mercury observed in the yolk was much smaller than could be expected from the electropherograms A heterogenity of the α globulin fractions seems possible and maybe one part is involved in the production of yolk and another part in the production of egg white Whether this is true or not the association of methyl mercury to the egg white proteins must be much stronger than to the yolk precursors

Up to the present it has not been possible to find a satisfying explanation to the different localization of mercury in the plasmas of the cocks and of the hens respectively A very high association constant for the proteins participating in the formation of yolk seems possible

bound to the plasma proteins than to the blood corpuscles The methyl mercury, on the other hand, preferentially accumulated in the blood corpuscles, as indicated by a high ratio The great uptake of methyl mercury in the blood corpuscles may be correlated to their high contents of sulphydryl groups (WEISSMAN *et al* 1950) and to the affinity of methyl mercury for thiols (BAHR & MOBERGER 1954) It has also been demonstrated that ethyl mercury, once entered an erythrocyte and bound to the hemoglobin, unwillingly leaves it (TAKEDA *et al* 1968), and a similar behaviour of methyl mercury seems reasonable

Injection of the phenyl and methoxyethyl mercurials presented an initially strong binding of mercury to the blood corpuscles, but with increasing survival of the birds, the ratio of mercury between blood corpuscles and plasma became similar to that seen after injection of inorganic mercury These changes are in consistence with a decomposition of the mercurials to inorganic mercury

In a previous chapter it was shown that methyl mercury more rapidly disappeared from the blood of the hen quails than from the blood of the cock quails This must be attributed to the great accumulation of methyl mercury in the oviduct and to the subsequent excretion in the white of the eggs Concerning the other mercurials these more rapidly left the blood of the cocks than the blood of the hens The concentration of mercury in the cock quail blood plasma, 1 hour after injection of mercuric nitrate, has been demonstrated to be 11 % of the concentration 5 minutes after injection, while in hen quails the corresponding value was 45 % (BACKSTRÖM unpublished observations) This must also be attributed to the egg production, but in this case to the formation of yolk The yolk proteins are synthesized in the liver and are then transported in the blood to the developing follicles In the yolk, however, the mercury was bound to the slowly migrating lipoproteins, while in the blood of the laying hens, it was bound to the electrophoretically more mobile α globulins No mercury was seen in the plasma lipoprotein fractions The lipoproteins seen in the yolk electropherograms seem identical to lipovitellin and lipovitellenin (FEVOLD 1951) and isolation of these fractions from eggs laid by hens injected with mercuric nitrate have demonstrated them to contain about 98 % of the mercury in the yolk (BACKSTRÖM unpublished observations) Almost all of the mercury was bound in the protein part and very little followed the lipids at extraction with ethanol According to VANSTONE *et al* (1955) there is a disappearence of the α_1 globulins (which seemed to carry the major part of the mercury) in the serum of laying hens It appears as if the mercury in the mercury loaded α globulins, in some way, combines with the lipids in the serum lipoproteins to yield the mercury loaded lipoproteins in the yolk A fusion in the follicle wall of the mercury carrying serum α globulins and the serum lipoproteins seems reasonable According to ROMANOFF & ROMANOFF (1949) the follicle wall mainly acts as a selective ultrafilter for the yolk constituents but a more active function seems possible The incorporation of mercury in the yolk lipoproteins may eventually be explained by their

(*TSURUGA* 1963, HANNERZ 1968) An increasing uptake in skeletal muscles and most other organs of pikes perorally given methyl mercury has, however, been reported (OHMOMO *et al* 1969) In fish from mercury-polluted lakes and rivers, JOHNELS *et al* (1967, 1968) detected the highest concentrations of mercury in heart and skeletal muscles whereas RAEDER & SNEKVIK (1941), in marine fish, observed the highest concentration in spleen followed by liver and kidneys

In the following are presented distribution studies of mercury in fish in various ways administered inorganic, phenyl and methyl mercury labelled with ^{203}Hg The information has been obtained by autoradiographic and scintillation counting techniques according to methods described above (cf chapter I)

MATERIALS AND METHODS

^{203}Hg labelled mercuric nitrate, phenyl mercuric nitrate and methyl mercuric nitrate were obtained from AB Atomenergi, Studsvik, Sweden The specific activity and the radiochemical purity was the same as for the compounds used in the previous avian experiments

The species of fish used were salmon (*Salmo salar*), speckled trout (*Salvelinus fontinalis*), pike (*Esox lucius*), pike perch (*Lucioperca lucioperca*) and perch (*Perca fluviatilis*) With the exception of the perches, which were caught in Lake Mälaren the fishes, aged ½—3 years, were obtained from fish-cultures. The fishes were housed in 100 litre aquaria with a continuous throughflow of fresh water The temperature of the water in the aquaria was 3—10° C.

Some of the experiments, in which the fishes were kept in model biotopes built up in large ponds were performed in collaboration with Dr L Hannerz, Institute of Freshwater Research Stockholm, Sweden

In the pond experiments the fishes (pikes) nourished from smaller fishes (pike perch) but in the aquarium experiments only the speckled trout accepted food (ox liver)

The experiments were performed according to the following plan

1 Twenty four salmons each weighing about 70 g, were injected intramuscularly in the longitudinal dorsal muscles or were given perorally the mercurials in a dose corresponding to 0 5 mg mercury/kg body weight Four fishes were used for each compound and mode of administration Whole body autoradiography was performed after survival times of 1 hour, 1 day, 3 days and 10 days

2 Thirty speckled trout, each weighing about 450 g, were injected intravenously with the phenyl and methyl compounds in a dose of 0 1 mg/kg and the inorganic mercury in a dose of 0 05 mg/kg calculated as mercury The injection was given in the duct of Cuvier in a few cases with the fish slightly anaesthetized with tricaine methanesulphonate (MS 222 Sandoz) Ten fishes were used for each compound and organs were sampled for analysis 1 hour, 1 day, 4 days, 10 days

CHAPTER SEVEN

Distribution of Mercury in some Fresh-Water Fishes

In 1965 and 1966, analyses of mercury in the Swedish aquatic environment revealed high values in fish, especially in industrially polluted waters (WESTERMARK *et al* 1965, JOHNELS *et al* 1967) It has been well known for several years that fish may accumulate mercury considerably more than other animals (STOCK & CUCUEL 1934, RAEDER & SNEKVIK 1941, 1949 a, b, RANKAMA & SAHAMA 1950) and the concentration factor in pike flesh has been determined to about 3,000 (JOHNELS *et al* 1967) The major part of the mercury in Swedish fish was methyl mercury (WESTÖÖ 1966 b, NOREN & WESTÖÖ 1967) and a methylation process was considered involved, since the waters often contained mercurials other than methyl mercury (JENSEN & JERNELÖV 1969)

Mercurials are considered highly toxic to fish (DOUDOROFF & KATZ 1953, ALABASTER 1958), but in spite of relatively high mercury content values no observations on toxic symptoms have been reported in Swedish fish In experimental conditions, on the other hand, toxic effects have been described by some investigators (CARPENTER 1927, BOETIUS 1960) The general conclusion has been that mercury primarily affects the skin and the gills of the fish coagulating the mucus covering and causing death by suffocation HANNERZ (1968) in his experiments with fish in mercury polluted ponds in some cases also observed a reduced vitality of the fishes and an incapability to withstand stress situations

The Minamata disease in Japan caused by the ingestion of fish contaminated with methyl mercurials, also presented symptoms in the fish (KURLAND 1960 TAKEUCHI 1968) Many of the poisoned fishes floated on the surface of the water and could easily be caught by hands The fishes were mostly thin and had lost their luster A prominent symptom was a cataract in the lens of the eye The cataract was characteristic only in piscine cases of Minamata disease and was never observed in mammals Pathological changes also occurred in the central nervous system of the fishes with degeneration of the nerve cells in various parts of the brain Histopathological changes, caused by methyl mercury experimentally fed to pikes, have also been described by MIETTINEN *et al* (1969) The most damaged organs were liver, kidneys and pseudobranch

Distribution studies of mercury in fish are few in numbers In experimental conditions, when the fishes have been studied during a relatively short time the highest concentrations usually have been found in kidneys spleen liver and gills and relatively low concentrations have been observed in skeletal and heart muscles



Fig 49 Autoradiogram of a young salmon 3 *days after ingestion of phenyl mercury* Large amounts of mercury are retained in the digestive tract but a high concentration is also observed in kidney *liver and gall bladder* Note the uptake in the choroid gland in the eye

kidney liver and myocardium and with increasing survival time there was an increased uptake of mercury in most organs At the longest survival time the highest concentration was seen in the caudal parts of the trunk kidney (opistonephros) in which the mercury localized irregularly rendering the kidney a spotty appearance In the anterior part of the kidney and in the head kidney (pronephros) the average mercury concentration was equal to that in the blood Some parts of *the head kidney however presented* a very high uptake A high concentration of mercury was also seen in the gills and in the pseudobranch In blood myocardium, liver *bile and spleen there was a moderate uptake Almost no mercury* was seen in brain or skeletal muscles

2 Ingestion of phenyl mercuric nitrate resulted in a more obvious absorption of mercury The distribution pictures much resembled those of inorganic mercury *Somewhat higher* concentrations of mercury however appeared in blood liver bile and skeletal muscles (Fig 49) The major part of the mercurial *seemed to* remain in the gastro intestinal tract even at the longest survival time studied

3 Methyl mercuric nitrate was quite readily absorbed after ingestion One hour after the peroral dosage the mercury concentration was high in blood and liver The skeletal muscles showed a flamed appearance with moderate *concentrations* irregularly distributed A marked uptake was seen in brain and spinal cord. One day later the amounts of mercury in the digestive tract had markedly decreased A high uptake was seen in liver spleen *kidneys gills* and pseudobranch The concentration was also high in blood and myocardium, *whereas comparatively low* concentrations appeared in brain and skeletal muscles *A high* activity was also observed in the pituitary

and 30 days after the injection Samples were taken from blood, trunk kidney (opistonephros), liver, bile, muscle (dorsal longitudinal), myocardium, spleen, brain (whole), gill and pseudobranch The concentration of mercury was calculated after analysis in a well type scintillation crystal (Packard), and the values, expressed as the concentration per gram of tissue in percentages of the injected dose per gram body weight, were plotted against the survival times

3 Twelve speckled trout, each weighing about 350 g, were injected intravenously with the mercurials in a dose corresponding to 0 5 mg mercury/kg Four fishes were used for each compound and whole body autoradiography was performed 1 hour, 1 day, 4 days and 10 days after the injection Due to the size of the fishes it was not possible to take sagittal whole body sections but instead transversal sections were cut through different regions

4 Nine perches were injected intramuscularly with the compounds in a dose corresponding to 0 5 mg mercury/kg Three fishes were used for each compound and autoradiography was performed 1 day, 5 days and 10 days after the injection

5 Pikes and pike perches which had been kept in fresh-water model biotopes added the mercurials via the water were obtained from Dr L Hannerz, Institute of Freshwater Research, Stockholm For a detailed description of the arrangements compare HANNERZ (1968) The following compounds were used

a Phenyl mercuric acetate in alcoholic solution was introduced in the water as a single dose The initial mercury concentration in the water was 0 01 mg/l and the activity of ^{203}Hg was 0 12 μCi/l

b Methyl mercuric hydroxide in water solution was continuously added the water to maintain a mean concentration of 0 003 mg Hg/l which corresponded to 0 14 μCi/l at the start of the experiment

c Mercuric chloride in weak hydrochloric acid solution was continuously added the water The mercury concentration in the water varied between 0 3 $\cdot$ 10^{-4} and 1 6 $\cdot$ 10^{-4} mg/l and the activity of ^{203}Hg at the peak concentration was 0 1 μCi/l

After living for 5 weeks (in the pond with mercuric chloride 10 weeks) the fishes were kept for a few days in tanks with a continuous throughflow of untreated fresh water and were then prepared for whole body autoradiography Two or three fishes were used from each pond

6 Microautoradiography was performed on some selected organs from speckled trout and pikes injected intravenously with ^{203}Hg labelled mercuric nitrate and methyl mercuric nitrate (0 5 mg Hg/kg) The survival times of these fishes were 1 day, 3 days and 7 days

RESULTS

Peroral administration

1 Mercuric nitrate ingested by the salmons was slowly absorbed from the digestive tract and 10 days after the dosage large amounts remained unabsorbed One hour after the dosage a slight uptake of mercury could be seen in blood

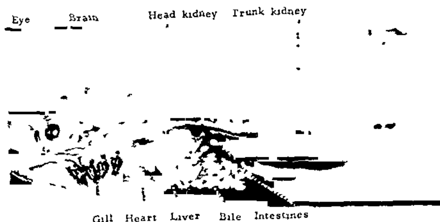


Fig 49 Autoradiogram of a young salmon 3 days after ingestion of phenyl mercury Large amounts of mercury are retained in the digestive tract but a high concentration is also observed in kidney liver and gall bladder Note the uptake in the choroid gland in the eye

kidney liver and myocardium and with increasing survival time there was an increased uptake of mercury in most organs At the longest survival time the highest concentration was seen in the caudal parts of the trunk kidney (opistonephros) in which the mercury localized irregularly rendering the kidney a spotty appearance In the anterior part of the kidney and in the head kidney (pronephros) the average mercury concentration was equal to that in the blood Some parts of the head kidney however presented a very high uptake A high concentration of mercury was also seen in the gills and in the pseudobranch In blood myocardium liver bile and spleen there was a moderate uptake Almost no mercury was seen in brain or skeletal muscles

2 Ingestion of phenyl mercuric nitrate resulted in a more obvious absorption of mercury The distribution pictures much resembled those of inorganic mercury Somewhat higher concentrations of mercury however appeared in blood liver, bile and skeletal muscles (Fig 49) The major part of the mercurial seemed to remain in the gastro intestinal tract even at the longest survival time studied

3 Methyl mercuric nitrate was quite readily absorbed after ingestion One hour after the peroral dosage the mercury concentration was high in blood and liver The skeletal muscles showed a flamed appearance with moderate concentrations irregularly distributed A marked uptake was seen in brain and spinal cord One day later the amounts of mercury in the digestive tract had markedly decreased A high uptake was seen in liver spleen kidneys, gills and pseudobranch The concentration was also high in blood and myocardium, whereas comparatively low con entrations appeared in brain and skeletal muscles A high activity was also observed in the pituitary

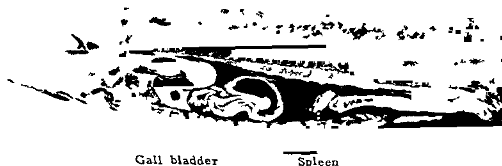


Fig 50 Distribution of mercury in a young salmon 10 days after ingestion of methyl mercury *Note the accumulation in the brain and in the skeletal muscles*

With increasing survival time of the fishes there was an increased uptake of mercury in brain and skeletal muscles Ten days after the methyl mercury intake the highest concentration of mercury appeared in the liver and in the gastrointestinal mucosa followed by the kidneys and the spleen (Fig 50) Most other organs presented a moderate, evenly distributed uptake The concentration in the blood was equal to the body average The concentration in the bile was low throughout the study

Parenteral administration

No conspicuous differences between the species were observed

1 Mercuric nitrate, intravenously injected, distributed in conformity with that injected intramuscularly The highest concentrations were reached in the kidneys and in the spleen followed by the liver (Fig 51 52 55) The concentration changes with time were relatively small

The blood concentration remained relatively high throughout the study In the myocardium considerably more mercury localized than in the skeletal muscles The mercury in the spleen was irregularly deposited and at longer survival times it seemed mainly to localize in the white pulp (Fig 51) In the central nervous system only small amounts of mercury were seen The strongest localization occurred in the walls of the cerebral vessels In the sympathetic ganglia there was a high concentration

The intestinal mucosa partially showed a high uptake of mercury A simultaneous biliary excretion made it difficult to ascertain a true intestinal excretion The liver reached a high concentration and seemed to increase its uptake throughout the study In the pancreas only moderate amounts of mercury appeared Moderate to high concentrations of mercury were seen in the gills No preferential binding site was observed the gill filaments showed an even uptake in all parts The pseudobranch seemed to accumulate less mercury than the gills



Fig 51 Distribution of mercury in a young salmon 3 days after an intramuscular injection of mercuric nitrate The dominating uptake of mercury is seen in the kidney Note the spotted appearance of the spleen

Inorganic mercury strongly accumulated in the piscine thyroid No uptake was observed in the pituitary whereas moderate concentrations were seen in the components of the adrenal system including the corpuscles of Stannius (Fig 56) The renal uptake of mercury exceeded that of any other organ The parenteral administration resulted in a more pronounced uptake in the cranial parts than was seen after ingestion Microautoradiography of the opistonephros (speckled trout) revealed the mercury to concentrate in certain regions of the proximal convoluted tubules probably the terminal parts (Fig 60) No mercury could be observed in the glomeruli The male as well as the female gonads moderately accumulated mercury in their interstitial tissue A rather high uptake was seen in the oviduct and in the follicular walls whereas no or very little mercury could be observed in the eggs (Fig 56) In atretic follicles however there was a high uptake No mercury could be observed in the contents of the testicular lobules

The skeletal muscles concentrated rather little mercury Due to a pronounced uptake in the tissues between the myomeres these were distinctly outlined

A strong uptake of mercury was seen in the choroid gland of the eye

2 Phenyl mercury intramuscularly injected was badly absorbed The absorbed portion distributed in agreement with the intravenously administered phenyl mercury The highest concentrations were seen in the kidneys and in the spleen followed by the liver (Fig 53 61 62) As was the case after injection of inorganic mercury the distribution patterns changed rather slowly during the observation period

The blood concentration slowly declined One day after the i v injection it was 80 % of the 1 hour concentration and after 10 days 35 % of this value (Fig 53) The myo ardium presented a moderate up ake except in its cortical lymphoid

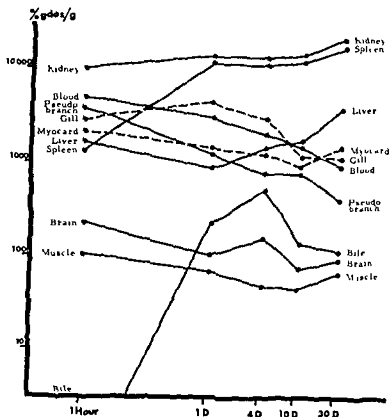


Fig 52 Concentration of mercury in some tissues of speckled trout injected i v with mercuric nitrate Each value represents the mean of two fishes

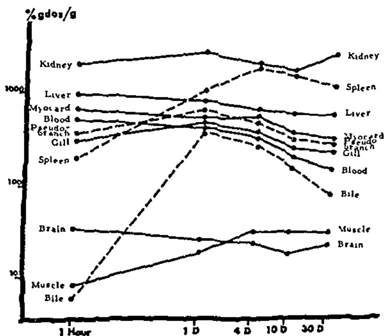


Fig 53 Concentration of mercury in some tissues of speckled trout injected i v with phenyl mercuric nitrate Each value is the mean of two fishes

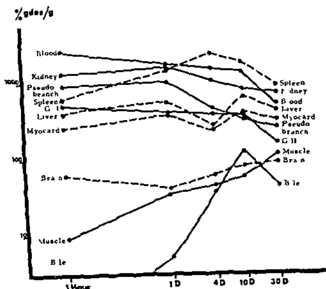


Fig 54 Concentration of mercury in some tissues of speckled trout injected i v with methyl mercuric nitrate Each value represents two fishes

muscle layer where there was a very high uptake (Fig 62) The spleen strongly accumulated mercury but the spotted uptake seen after injection of inorganic mercury never appeared In the central nervous system only small amounts of mercury were observed

The gastro intestinal mucosa accumulated considerably more mercury when phenyl mercury instead of inorganic mercury was injected The liver uptake was high and there was a marked biliary excretion (Fig 53, 61, 62) The delayed liver uptake seen after injection of mercuric nitrate, was not observed A pronounced uptake and retention of mercury occurred in the wall of the gall bladder (Fig 62)

The pseudobranch at all times studied showed a much higher uptake than the true gills (Fig 53)

The pituitary concentration of mercury was equal to that in the blood A very high uptake with a marked retention was observed in the epiphysis The corpuscles of Stannius showed a moderate uptake

The renal distribution was very similar to that of inorganic mercury Only a low uptake of mercury was seen in the germinal cells while there was a moderate concentration in the gonadal interstitium.

The skeletal musculature showed a relatively low concentration in the white muscles whereas there was a pronounced uptake in the red muscles along the lateral line (M lateralis superficialis trunci) and in the fin muscles (Fig 61,

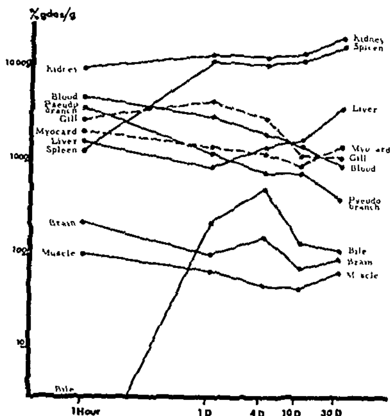


Fig 52 Concentration of mercury in some tissues of speckled trout injected i v with mercuric nitrate Each value represents the mean of two fishes

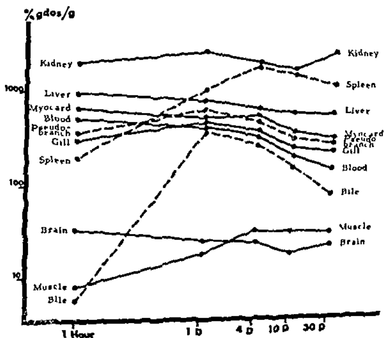


Fig 53 Concentration of mercury in some tissues of speckled trout injected i v with phenyl mercuric nitrate Each value is the mean of two fishes



Fig 56 Autoradiogram (detail) of a speckled trout 10 days after an i v injection of mercuric nitrate



Fig 57 Accumulation of mercury in the pseudobranch of a perch 5 days after an i m injection of methyl mercury

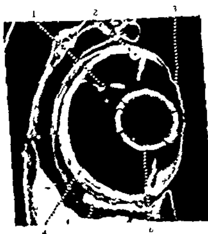


Fig 58 Distribution of mercury in the eye of a salmon 10 days after an i m injection of methyl mercury Note the accumulation in the lens capsule

1 falciform process 2 iris 3 cornea 4 retina 5 choroid gland 6 lens



Fig 59 Distribution of mercury in the brain of a pike perch kept for 5 weeks in water polluted with methyl mercury Note the high uptake in the midbrain

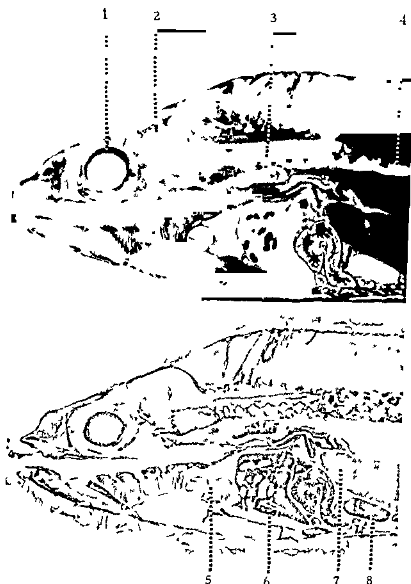


Fig 55 Autoradiogram (top) and corresponding section of a perch 5 days after an i m injection of mercuric nitrate High concentrations of mercury appear in kidney liver and spleen
1 eye 2 brain 3 head kidney, 4 trunk kidney 5 heart 6 liver 7 testis 8 spleen

62) In the eye a high concentration was seen in the choroid gland whereas other parts showed a low uptake

3 Methyl mercury, parenterally administered also showed relatively small chronological changes in its distribution When intramuscularly injected it was slowly absorbed and reached its highest concentration in the liver Intravenously injected, on the other hand it reached its highest concentration in spleen kidneys and blood (Fig 54)



Fig 56 Autoradiogram (detail) of a speckled trout 10 days after an i v injection of mercuric nitrate



Fig 57 Accumulation of mercury in the pseudobranch of a perch 5 days after an i m injection of methyl mercury

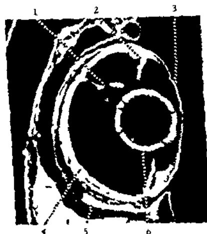


Fig 58 Distribution of mercury in the eye of a salmon 10 days after an i m injection of methyl mercury Note the accumulation in the lens capsule
1 falciform process 2 iris 3 cornea 4 retina 5 choroid gland 6 lens



Fig 59 Distribution of mercury in the brain of a pike perch kept for 3 weeks in water polluted with methyl mercury Note the high uptake in the midbrain



Fig 60 Microautoradiogram of the kidney from a speckled trout 3 days after an i v injection of mercuric nitrate The mercury (black grains) is preferentially localized in the terminal parts of the proximal tubules x 64

One hour after the i v injection the blood showed the highest uptake of mercury The blood concentration then remained high throughout the study The 10 days value was 40 % and the 30 days value was 20 % of the 1 hour s value (Fig 54) The myocardium accumulated considerably more mercury than the skeletal muscles An intense uptake with an even distribution was seen in the spleen Microautoradiography revealed the mercury mainly to localize in the splenic cords The brain and the spinal cord rapidly reached a marked concentration which then slowly increased Within the brain the mercury was rather evenly distributed

The gastro intestinal mucosa presented high concentrations of mercury throughout the study The liver reached high values and there was a slight excretion of mercury in the bile The accumulation in the wall of the gall bladder was less prominent than after injection of phenyl mercury

The gills showed moderate concentrations of mercury which followed those in the blood The pseudobranch strongly accumulated mercury (Fig 57)

The pituitary concentrations of mercury equalled those in the blood In the epiphysis there was a very high uptake The thyroid uptake was low

The renal uptake was relatively even. Some tubules showed a slightly higher uptake than others.

Moderate concentrations of mercury appeared in the gonads.

The uptake in the germinal cells was low.

The skeletal muscles showed an increasing concentration throughout the study. The red muscles accumulated considerably more than the white muscles.

In the eye there was a relatively high uptake of mercury in the retina as well as in the lens capsule (Fig. 58). High concentrations were also seen in the falciform process and in the iris. The choroid gland showed a very high accumulation.

Pond experiments

1. The highest concentrations of methyl mercury were observed in the kidneys and in the pseudobranch (Fig. 63). In the pronephros and in the cranial part of the opistonephros there was a comparatively low uptake. The major renal uptake was observed in the caudal parts.

Relatively high concentrations of mercury were seen in the liver and in the gastro-intestinal mucosa. The liver uptake was very irregular. Moderate concentrations appeared in spleen, myocardium, gills and brain. Some cerebral regions, especially the nerve-cell rich part of the midbrain forming the bottom of the third ventricle, showed an uptake markedly exceeding that of other regions (Fig. 59). A marked accumulation of mercury was also seen in the skeletal muscles.

2. Only pikes were autoradiographed from the pond with phenyl mercury. When the fishes were prepared, an abnormal, green colour of the flesh and of the liver was seen. The dominating uptake of mercury was presented by the liver, which had a concentration exceeding that of any other organ (Fig. 64). A high uptake was also seen in the pseudobranch and in the kidneys, including the head kidney. Moderate concentrations of mercury appeared in the spleen, in the intestinal mucosa and in the gills. The pyloric part of the stomach seemed to concentrate more mercury than other parts. The skeletal and heart muscles had a comparatively low uptake. The pikes seemed to accumulate more mercury in their musculature than the pike perches, as illustrated by Fig. 64, where no mercury can be observed in the pike perch localized in the stomach of the pike.

3. The pikes which had lived for 10 weeks in the pond contaminated with mercuric chloride showed their highest uptake of mercury in the pseudobranch and in some renal tubules. The remaining kidney and the liver had a relatively high uptake. A somewhat lower concentration was seen in the spleen, in which the mercury distributed evenly. A marked uptake was also observed in brain, gills, intestinal mucosa, skeletal muscles, myocardium and lens capsule. No mercury was visible in the blood. A high uptake was also observed in the mucosa of the olfactory pit.



Fig 60 Microautoradiogram of the kidney from a speckled trout 3 days after an i v injection of *mercuric nitrate* The mercury (black grains) is preferentially localized in the terminal parts of the proximal tubules x 64

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Moderate concentrations of mercury appeared in the gonads

The uptake in the germinal cells was low

The skeletal muscles showed an increasing concentration throughout the study The red muscles accumulated considerably more than the white muscles

In the eye there was a relatively high uptake of mercury in the retina as well as in the lens capsule (Fig 58) High concentrations were also seen in the falciform process and in the iris The choroid gland showed a very high accumulation

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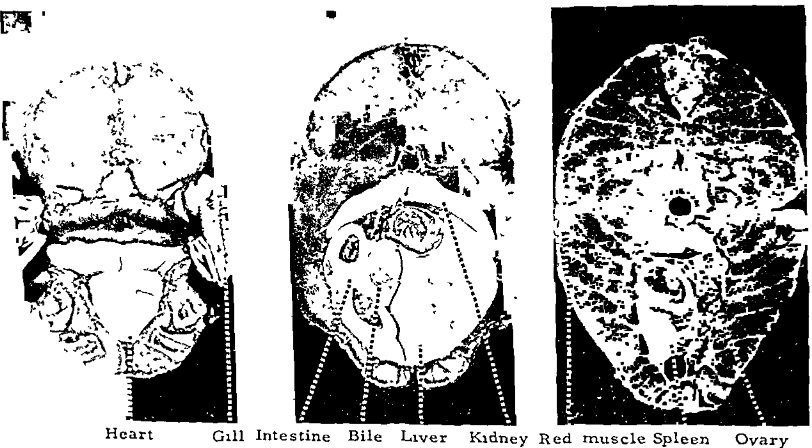


Fig 61 Distribution of mercury in a speckled trout 1 day after an i v injection of phenyl mercury Note the high uptake in the red flesh as compared with the white flesh



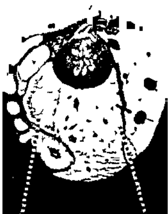




Fig 62 Distribution of mercury in a speckled trout 10 days after an i v injection of *phenyl mercury* Note the retention in the cortical layer of the myocardium and in the wall of the gall bladder



Fig 63 Autoradiogram of a *pike kept for 5 weeks* in a pond polluted with methyl mercury Note the accumulation in the *pseudobranch* and in the caudal part of the trunk kidney

DISCUSSION

When interpreting the results the great variation between different species of *fish* concerning anatomy physiology metabolism and behaviour must be re *membered* The present study has been limited to a few species of fresh water fish and generalizations about the mercury distribution in fish must therefore be *regarded with* cautiousness Since no greater differences in mercury uptake and *distribution* have been observed between the species used in the *experiments the* results will be considered without *reference to the species*

The most striking observation in the piscine experiments was the slow kinetics of mercury once distributed in the body The quantitative data were considered unsufficient to be the basis of calculations of the biological half life of the mer curials but they suggest a long half life HANNERZ (1968) estimated the biological half life of phenyl mercury in pike perch to about 70 days and TIL LANDER (1969) reported that the half life of methyl mercury in pike varied between 25 and 600 days

The slow metabolism of the mercurials in fish as compared with birds or mammals may be attributed to the much lower basal metabolism in fish This decreases markedly during starvation but in the experiments the speckled trout willingly accepted food The temperature in the water was low but the metabolic rate in speckled trout which is comparatively high changes little from 5° C to 20° C (FRY 1957)

A greater occurrence of mercury binding groups in fish than in birds or mammals might also contribute Fish muscles contain somewhat more methionine and cysteine than the muscles of other animals (BEVERIDGE 1947) Fish is also richer in nitrogen (HAMOIR 1955) and mercurials especially methyl mercury have a strong affinity for amides and amines (HUGHES 1957)

The observation by JOHNELS *et al* (1967) that mercury contaminated Swedish fish had the highest concentration of mercury in the heart muscle followed by the axial musculature has not been confirmed They however analysed fishes that

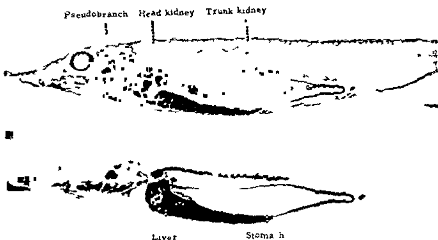


Fig 64 Histolog cal section (top) and corresponding autoradiogram of a pike kept for 5 we ks in water polluted with phenyl mercury Note the high concentration of mercury in the liver the kidney and the pseudobranch The pike perch localized in the stomach of the p ke has concentrated less mercury than the pike

had been exposed to mercury for a relatively long time The quantitative data in the present investigation also indicated an increasing uptake in the skeletal muscles The reason why the red muscles and the cortical layer of the myocardium accumu lated more mercury than other muscles is uncertain The red muscles however, have a higher metabolism and are richer in sarcoplasm than the white muscles (HAMOIR 1955) The green colour of the flesh of the pikes in the pond with phenyl mercury may be attributed to a deposition in the tissues of bile pigments su h as biliverdin Histopathological examination of the liver only demonstrated excess bile in the bile ducts The green colour of the pikes in the present investiga tion may be related to similar observations on wild-captured pikes submitted to the National Veterinary Institute Stockholm Sweden (LJUNGBERG personal communication)

Though the principal distribution of mercury in fish resembled that in birds and mammals certain discrepancies were noted The latter animals did not show the high uptake of mercury in the spleen which was observed in the fishes. Piscine erythrocytes are very fragile (BLACK 1951) and a mercury induced damage would result in an increased phagocytosis by the macrophages in the splenic cords The mercurials especially methyl mercury also strongly bound in the blood and the microautoradiograms of methyl mercury displayed the highest splenic uptake in the splenic cords

Mercury localized very unevenly in the kidneys The caudal opistonephros showed the highest uptake whereas less occurred in the middle region The cranial

part and the pronephros, as a rule, showed a comparatively low uptake The lower uptake in the anterior opistonephros may be correlated to the absence of functioning nephrons in this region (HARDER 1964) The pronephros mainly consists of a lymphoid reticulum and has no excretory function In the histological sections single nephrons were, however, observed in this part, and these seemed to be responsible for the areas with a high uptake of mercury which were seen on the autoradiograms

In the functioning kidney the inorganic mercury selectively concentrated in certain regions probably in the terminal parts of the proximal tubules A high uptake of mercury in corresponding region has also been observed in the mammalian nephron (BERGSTRAND *et al* 1958)

The marked accumulation and retention of mercury in the wall of the gall bladder is interesting In the gall bladder the bile is concentrated by the absorption of salts and water and a disturbance of this function could explain that the gall bladder of the experimental fishes, as compared with untreated, often was strongly filled with bile

Irrespective of the mode of administration a relatively high concentration of mercury appeared in the gills An interesting analogy between gill and kidney tubule action has been presented by MEYER (1952) In the kidney the mercuric ion produces diuresis by disturbing the sodium chloride and water reabsorption MEYER demonstrated the mercuric ion to inhibit the active uptake of sodium in the gills of gold fish resulting in an increased sodium loss The uptake of sodium via the gills is of prime importance for many fresh-water fishes, and an impairment of this function may be very harmful A fresh water fish is hypertonic to the surrounding water and is thus continuously faced with the need to dispose of the water it absorbs osmotically and to replace the salts lost by diffusion and excretion The salts will be taken up from the food or from the water via the gills During spawning and migration many species undergo long periods of fasting and will then replace their salt loss via the gills only An inhibition of this uptake might explain the greater toxicity of heavy metals for fresh water fish than for marine species (DOUDOROFF 1957) the latter having no need to absorb salts via the gills

The pronounced uptake and retention of mercury in the pseudobranch is interesting The function of this organ is very uncertain and many theories have been put forward The suggestion of PARRY & HOLLIDAY (1960) that the pseudobranch produces or activates a hormone affecting the chromatophores is interesting Removal of the pseudobranch was followed by a darkening of the fish due to a chromatophore expansion A darkening of fish has also been reported after mercury exposition (HANNERZ 1967) Degenerative changes in the pseudobranch after exposition to methyl mercury have been described by MIETTINEN *et al* (1969) Among other theories, recent observations on the function of the pseudobranch as a baro and chemoreceptor (LAURENT 1967) should be mentioned



Fig 65 Detail from an autoradiogram of a pregnant mouse 4 days after an i v injection of methyl mercury Note the intense accumulation of mercury in the lens of the fetus (BACKSTRÖM unpublished)

since mercury induced disturbances of the osmoregulatory mechanisms would be very harmful to the fish

The strong uptake of mercury in the choroid gland is interesting in this connection It was suggested by PARRY & HOLLIDAY (1960) that this organ controls the entry of the chromatophore inhibiting hormone into the general circulation, and a multiple action of mercury on the pseudobranch choroid gland system seems possible

The uptake of mercury in the lens of the fish eye may be correlated to the cataract observed in piscine cases of Minamata disease (cf above) The lens is rich in sulphydryl groups (BARRNETT 1953) and a sulphur mercury binding seems probable The lens of the quail eye also accumulated mercury and in mice a very strong uptake of methyl mercury has been demonstrated in the fetal as well as in the maternal lens (BACKSTRÖM unpublished Fig 65) Also in man there are many reports concerning an uptake of mercury in the lens (ATKINSON 1943, BURN 1962)

In the gonads moderate concentrations of mercury appeared in the interstitial tissues and in the follicular envelope whereas no or little mercury was seen in the germinal cells The strong uptake seen in the avian yolk was not observed in the yolk of the fish oocytes The reason for this is uncertain, maybe differences in the rate of yolk formation are important The localization in the fish testicles is interesting from a special point of view The kinetics of mercury involves a biological conversion of inorganic mercury to methyl mercury It has been demonstrated that this is performed by certain bacteria (cf above) A pronounced capacity of fish testicles to methylate certain substances has recently been described

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The marked accumulation and retention of mercury in the wall of the gall bladder is interesting In the gall bladder the bile is concentrated by the absorption of salts and water and a disturbance of this function could explain that the gall bladder of the experimental fishes, as compared with untreated often was strongly filled with bile

Irrespective of the mode of administration a relatively high concentration of mercury appeared in the gills An interesting analogy between gill and kidney tubule action has been presented by MEYER (1952) In the kidney the mercuric ion produces diuresis by disturbing the sodium chloride and water reabsorption MEYER demonstrated the mercuric ion to inhibit the active uptake of sodium in the gills of gold fish resulting in an increased sodium loss The uptake of sodium via the gills is of prime importance for many fresh water fishes and an impairment of this function may be very harmful A fresh water fish is hypertonic to the surrounding water and is thus continuously faced with the need to dispose of the water it absorbs osmotically and to replace the salts lost by diffusion and excretion The salts will be taken up from the food or from the water via the gills During spawning and migration many species undergo long periods of fasting and will then replace their salt loss via the gills only An inhibition of this uptake might explain the greater toxicity of heavy metals for fresh water fish than for marine species (DOUDOROFF 1957) the latter having no need to absorb salts via the gills

The pronounced uptake and retention of mercury in the pseudobranch is interesting The function of this organ is very uncertain and many theories have been put forward The suggestion of PARRY & HOLLIDAY (1960) that the pseudobranch produces or activates a hormone affecting the chromatophores is interesting Removal of the pseudobranch was followed by a darkening of the fish due to a chromatophore expansion A darkening of fish has also been reported after mercury exposition (HANNERZ 1967) Degenerative changes in the pseudobranch after exposition to methyl mercury have been described by MIETTINEN *et al* (1969) Among other theories recent observations on the function of the pseudobranch as a baro and chemoreceptor (LAURENT 1967) should be mentioned

Summary

The discovery of mercury as an environmental pollutant in Sweden, especially in the avian and piscine fauna, has been the motive for an investigation of the distribution of experimentally administered mercurials in the tissues of birds and fishes. Such information has been considered to be of great value in the understanding of the manifested and potential risks for these animals when contaminated with mercury. *Autoradiography*, scintillation counting and zone electrophoresis have been applied in the experiments. Using Japanese quail, a suitable pilot animal for birds, and several species of fresh water fish, the localization of mercury has been followed after parenteral or peroral administration of methyl mercury, phenyl mercury, methoxyethyl mercury and inorganic mercury (Hg^{2+}). The mercurials have been labelled with ^{203}Hg. In some avian experiments the stability of the carbon-mercury bond in methyl and phenyl mercury has been examined by comparative studies of the compounds double labelled with ^{203}Hg and ^{14}C. The experiments have yielded many data of which a few will be summarized.

The way of administration was of minor importance for the final, qualitative, distribution of mercury. Methyl mercury was readily absorbed after ingestion while the other compounds were less well absorbed.

The methyl mercury radical was found to be very stable in the avian body, whereas the phenyl mercury was rapidly decomposed to inorganic mercury.

The distribution pictures of methyl mercury in the quails markedly differed from those of the other mercurials, the latter presenting great similarities with each other. Methyl mercury was characterized by an even distribution of mercury in most organs including the kidneys, and by a slow excretion. In the hen quails the excretion was, however, more rapid than in the cock quails, which could be attributed to a pronounced concentration of methyl mercury in the white of the eggs. The avian oviduct also showed an uptake of methyl mercury exceeding that of most other tissues.

After injection of methyl mercury the brain slowly reached a high concentration of mercury. Injection of the other mercurials only resulted in a small cerebral uptake of mercury. Administration of these compounds resulted in more differentiated distribution patterns, and there was a more rapid excretion of mercury. There was also a pronounced accumulation of mercury in the yolk of the eggs while almost no mercury appeared in the white. Along with the yolk, the mercury from the phenyl, methoxyethyl and inorganic mercurials had the kidneys

(HOFMEISTER 1964), and it is tempting to wonder, whether this could contribute to the methylation of mercury

The irregular localization in the brain of methyl mercury in the pond experiment may be correlated to the observation by HANNERZ (1967) of balance disturbances in these fishes According to several authors (cf BROWN 1957) damage to the deeper parts of the midbrain also results in rolling and circus movements

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The results of the autoradiography and of the electrophoresis evoked the assumption of steric factors and the different origin of the yolk and egg white proteins as the reason for the different distribution of the various mercurials in egg white and yolk respectively

In the piscine experiments, the most outstanding feature was the slow kinetics of mercury once distributed No greater differences in the distribution of mercury were observed between the species The differences between the various compounds were less prominent than in the avian experiments The principal distribution otherwise showed many similarities with corresponding patterns in the birds Certain differences were however, noted The thyroid and the spleen e g demonstrated a much higher uptake of mercury in the fishes than in the birds Injection of phenyl mercury in the fishes also resulted in a strong retention of mercury in the wall of the gall bladder and in the cortical layer of the myocardium These localizations of mercury were not observed in the birds

The fishes obviously were faced with great difficulties to get rid of mercury once deposited After injection of methyl mercury there was a steadily increasing uptake of mercury in the muscles and in the brain throughout the study Injection of the inorganic mercurial resulted in a similar increasing uptake in kidneys, spleen and liver

A pronounced difference was observed between the white and the red flesh, the latter accumulating considerably more mercury than the former

Phenyl mercury strongly accumulated in the piscine liver, which may be related to a green colour presumedly caused by bile pigments, which appeared in the flesh of pikes kept in water defiled with phenyl mercury

A high uptake of mercury was noted in the gills and in the pseudobranch The significance of these localizations is uncertain but damage to these organs in fishes poisoned by mercurials have been reported

and the liver as their main target tissues Even now certain differences in the distribution of mercury could be observed between the sexes These differences however, were less apparent than after injection of methyl mercury Administration of methyl mercury also resulted in a more prolonged retention of mercury in the blood of the cocks than in the blood of the hens whereas administration of the other compounds favoured a retention in the blood of the females.

The dose level was shown to be of importance for the relative excretion via the eggs An increased dosage of the phenyl, methoxyethyl and inorganic mercurials resulted as well totally as relatively in more mercury being excreted via the eggs As for the methyl mercurial a larger dose only slightly increased the excretion, and obviously the maximum excretory capacity had been reached already with the smaller dose

The plumage and other keratinized structures strongly concentrated mercury irrespective of the mercurial given, and this seems to be an important route of excretion of mercurials, especially methyl mercury The retention of methyl mercury in the body also resulted in a more continuous uptake of mercury in the feathers than was seen when the other mercurials were given

A pronounced concentration and retention of mercury was observed in the avian anterior pituitary when the phenyl, methoxyethyl and inorganic mercurials had been administered Certain clinical observations on mercury intoxication may also suggest an impaired function of the pituitary No accumulation of mercury has been observed experimentally in the mouse pituitary but there are some observations of high concentrations in human pituitaries

Depending on the type of mercurial, accumulations of mercury were observed in the adrenal medullary cells in the pancreas in certain nuclear areas in the brain in ganglia, in the germinal discs and in the epididymis The capacity of the mouse testis to concentrate inorganic mercury (Hg^{2+}) could not be reproduced in the quail

Though many signs pointed at a decomposition of phenyl and methoxyethyl mercury to inorganic mercury, certain differences appeared between the distribution pictures of these compounds especially in birds studied short times after administration Shortly after injection of the phenyl and methoxyethyl mercurials the Hg ratio between blood corpuscles and blood plasma also was similar to that seen after injection of the methyl mercurial, but after longer survival times it was more similar to that after injection of the inorganic mercurial This finding is in consistence with a decomposition of the two organo mercurials to inorganic mercury

The electrophoresis, combined with autoradiography and isotope scanning of the avian blood proteins demonstrated marked sex differences in the localization of mercury Electrophoresis was also run with yolk and egg white samples from mercury contaminated eggs, which resulted in characteristic localizations of mercury depending on the type of mercurial

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References

Ahlmark A Poisoning by methyl mercury compounds *Brit J Ind Med* 1948 *5* 117—119

Alabaster J S Toxicity of weedkillers algicides and fungicides to trout *Proc 4th Brit Weed Control Conf* 1958 1—2

Almquist J Some notes on the history of mercury intoxication *Acta Med Scand* 1929 *0* 464—476

Anson M L The sulfhydryl groups of egg albumin *J Gen Physiol* 1941 *24* 399—421

Atkinson W S A coloured reflex from the anterior capsule of the lens which occurs in mercurialism *Am J Ophthal* 1943 *26* 685—688

Bahr G F & G Moberger Methyl mercury chlorides as a specific reagent for protein bound sulfhydryl groups *Exptl Cell Res* 1954 *6* 506—518

Barrnett R J The histochemical distribution of protein bound sulfhydryl groups *J Nat Cancer Inst* 1953 *13* 905—925

Barrnett R J & A M Seligman Histochemical demonstration of protein bound sulfhydryl and disulfide groups of proteins *J Nat Cancer Inst* 1954 14 769—803

Berg, W A Johnels B Sjostrand & T Westermark Mercury content in feathers of Swedish birds from the past 100 years *Oikos* 1966 *17* 71—83

Berglund F & A Wretlind Toxikologisk värdering av kvicksilverhalter i svensk fisk *Vår Föda* 1967 *1* 10—11 (In Swedish)

Bergstrand A L Friberg & E Odeblad Localization of mercury in the kidneys after subcutaneous administration *A M A Arch Ind Health* 1958 17 253—256

Berlin M Renal uptake excretion and retention of mercury A study in the rabbit during infusion of methyl and phenylmercuric compounds *Arch Environ Health* 1963 6 626—633

Berlin M & S Ullberg Accumulation and retention of mercury in the mouse
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Arch Environ Health 1963 *6* 589—616

Berlin M J Fazackerley & G Nordberg Brains of mammals exposed to mercury vapours and to mercuric salts *Arch Environ Health* 1969 *18* 719—729

Beveridge J M R Sulphur distribution in fish flesh proteins *J Fisheries Research Board Canada* 1947 *7* 51—54

Bidstrup P L *Toxicity of mercury and its compounds* Elsevier New York 1964

Black E C Respiration in fishes *Univ Toronto Studies biol ser 59 Publ Ontario fish res lab* 1951 91—111

Boetius J Lethal action of mercuric chloride and phenylmercuric acetate on fishes *Medd Danmarks Fiskeri og Havundersøgelser* 1960 *3* 93—115

Booer J R The behaviour of mercury compounds in soil *Ann Appl Biol* 1944 *31* 340—358

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Ahlmark A Poisoning by methyl mercury compounds *Brit J Ind Med* 1948 *5* 117—119

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Grarzow J Pathologische Anatomie der endokrinen Drusen bei der Sublimatvergiftung Z Ges Exp Med 1926 49 487—499

Groebbels F Die Wirkung von Kleinhirnlasionen und die anatomisch physiologische Analyse Arch Physiol (Pflugers) 1929 221, 14—40

Guljarowsky W & A Winokuroff Zur Klinik und pathologischen Anatomie der Quecksilbervergiftungen Z des Neurol Psychiat 1929 121 1—23

Hagen U Toxikologie organischer Quecksilberverbindungen Naunyn Schmiedebergs Arch exp Path Pharmak 1955 224 193—205

Halasz, P F Mechler O Feher & S Damjanovich The effect of SH inhibitors on ganglionic transmission in the superior cervical ganglion of the cat Acta Physiol Acad Sci Hung 1960 18 47—55

Hammarstrom L L E Appelgren & S Ullberg Improved method for light microscopy autoradiography with isotopes in watersoluble form Exptl Cell Res 1965 37 608—613

Hamoir G Fish Proteins In Advances in protein chemistry 1955 10 227—288 Acad Press New York

Hannerz, L Anrikningen av metylkvicksilverhydroxid och fenylkvicksilveracetat i sotvattens biotopmodeller Rep to Royal Commission of Natural Resources Stockholm 1967 (In Swedish)

Hannerz, L Experimental Investigations on the Accumulation of Mercury in Water Organisms Rep Inst Freshw Res 1968 48 120—176 Stockholm, Sweden

Hansson T Diskussionsinlagg In Kvicksilverfrågan i Sverige Stockholm 1965 159—162 (In Swedish)

Harder W Anatomie der Fische Schweizerbartsche Verlagsbuchhandlung, Stuttgart, 1964

Helminen, M E Karppanen & K Henriksson Effect of a seed dressing containing methoxyethyl mercury silicate on pheasants Suomen Eläinlääkärilehti 1966, 72 381—389 (In Swedish)

Herlant M J Benoit A Tixier Vidal & I Assenmacher Modifications hyperphysaires au cours du cycle annuel chez le canard Pekin Comp Rend Acad Sci Paris 1960 250 2936 2938

Hesse E Versuche zur Therapi der Quecksilbervergiftung Arch exp Path Pharmak 1925 107 43—68

Hirashima K Untersuchungen uber den Einfluss des Quecksilbers auf gezuchtete Gewebe Folia Pharmacol Japon 1934 19 76—77

Hoare J L & J B Speakman Action of mercuric chloride on strained keratin Nature 1963 199 1091—1092

Hofmeister F Uber Methylierung im Tierkorper Arch exp Path Pharmak 1964 33 198—215

Hughes W L Jr An albumin fraction isolated from human plasma as a crystalline mercuric salt J Am Chem Soc 1947 69 1836—1837

Hughes W L Protein mercaptides Cold Spring Harbor Symp Quant Biol 1950 14 79—84

Hughes W L A physicochemical rationale for the biological activity of mercury and its compounds Ann New York Acad Sci 1957 65 454—460

Hunter D R R Bomford & D S Russell Poisoning by methyl mercury compounds Quart J Med 1940 33 193—213

Hunter D The Diseases of Occupations English Univ Press London 1955

Borg K Inverkan av betat utsade på viltfaunan *Proc VIII Nord veterinärmotet*, Helsingfors, 1958 (*In Swedish*)

Borg K, H Wanntorp, K Erne & E Hanko Kvicksilverförgiftningar bland vilt i Sverige *Rep State Veterinary Medical Institute*, Stockholm 1965 (*In Swedish*)

Borg, K, H Wanntorp, K Erne & E Hanko Mercury poisoning in Swedish wildlife *J Appl Ecol* 1966, Suppl 3 171—172

Borg, K Landfaunan och de kvicksilverhaltiga betningsmedlen *Fauna och Flora* 1968, 5 186—189 (*In Swedish*)

Borg, K, H Wanntorp, K Erne & E Hanko Alkyl mercury poisoning in terrestrial Swedish wildlife *Swedish Wildlife* 1969, *6*, 301—379

Brown, M E The Physiology of Fishes Acad Press, New York 1957

Bucker, E D. Paper electrophoretic pattern of human serum proteins compared with those of lower forms *Proc Soc Exptl Biol Med* 1960, *106*, 373—377

Burn, R A Mercurialentis *Proc Roy Soc Med* 1962, *55*, 322—326

Carpenter, K E The lethal action of soluble metallic salts on fishes *Brit J Exptl Biol* 1927, *4*, 378—391

Cassano, G., P L Viola, B Ghetti & L Amaducci The distribution of inhaled mercury (Hg^{203}) vapours in the brain of rats and mice *J Neuropath exp Neurol* 1969, *28* 308—320

Clarkson, T W Isotope exchange methods in studies of the biotransformation of organomercurial compounds in experimental animals *Rep Univ Rochester A E P* 1968, No UR—49—958

Common, R H, W P McKinley & W A Maw Filter paper electrophoresis of avian serum proteins *Science* 1953, *118*, 86—89

Cotter, L M Hazard of phenylmercuric salts *Occup Med* 1947, *4*, 305—309

Doudoroff, P & M Katz Toxicity of industrial wastes and their components to fish *Sewage Ind Wastes* 1953, *25*, 802—839

Doudoroff, P Water quality requirements of fishes and effects of toxic substances In M Brown The Physiology of Fishes Acad Press, New York 1957 pp 403—430

van Dyke, H B, B F Chow, V du Vigneaud, H L Fevold, G W Irving C N H Long, T Shedlovsky & A White Protein hormones of the pituitary body *Ann New York Acad Sci* 1943, *43* 253—426

Ellis, R W & S C Fang Elimination tissue accumulation and cellular incorporation and cellular incorporation of mercury in rats receiving an oral dose of ^{203}Hg labelled phenyl mercuric acetate and mercuric acetate *Toxicol Appl Pharmacol* 1967, *11* 104—113

Enders, A & Hr Noetzel Spezifische Veranderungen im Kleinhirn bei chronisher oraler Vergiftung mit Sublimat *Arch exp Path Pharmak* 1955, *225*, 346—351

Fevold, H Egg proteins In Advances in Protein Chemistry 1951, *6*, 187—252 Acad Press, New York

Friberg, L, E Odeblad & S Forssman Distribution of two mercury compounds in rabbits after a single subcutaneous injection *A M A Arch Ind Health* 1957, *16* 163—168

Fry, F E J The aquatic respiration of fish In M Brown The Physiology of Fishes Acad Press, New York 1957, pp 1—63

Gage, J C & A A B Swan The toxicity of alkyl and aryl mercury salts *Biochem Pharmacol* 1961, *8*, 77

Gage, J C Distribution and excretion of methyl and phenyl mercury salts *Brit J industr Med* 1964, *21* 197—202

Glick, D & B G Malmstrom Simple and efficient freeze-drying apparatus for the preparation of embedded tissue *Exptl Cell Res* 1952, *3*, 125—135

Martin W G K J Turner & W H Cook Macromolecular properties of vitellenin from egg yolk and its parent complex with lipids *Canad J Biochem Physiol* 1959, *37*, 1197—1207

Massmann W Beobachtungen beim Umgang mit *Phenylquecksilberbrenzkatechin Zbl Arbeitsmed* 1957, 7, 9—13

Meyer D K Effects of mercuric ion on sodium movement through the gills of gold fish *Federation Proc* 1962 *11*, 107

Miettunen V Y Ohmomo M Valtonen E Blankenstein K Rissanen M Tillander & J K Miettinen Preliminary notes on the distribution and effects of two chemical forms of methylmercury on pike Fifth R. I S Symp 1969 Helsinki Finland

Miller V L G E Bearse & K E Hammermeister Mercury retention A trait of chickens *Poultry Sci* 1959 a, *38* 1037—1039

Miller V L G E Bearse Y Kimura & E Csonka Strain differences in mercury retention by chicks *Proc Soc Exptl Biol Med* 1959 *b 100* 301—303

Miller V L P A Klavano & E Csonka Absorption distribution and excretion of phenylmercuric acetate *Toxicol Appl Pharmacol* 1960 2 344—352

Miller V L, D V Larkin, G E Bearse & C M Hamilton The effects of dosage and excretion of ethyl mercuric chloride *Toxicol Appl Pharmacol* 1961 *3*, 459—468

Miller V L D V Larkin G E Bearse & C M Hamilton The effects of dosage and administration of two mercurials on mercury retention in two strains of chickens *Poultry Sci* 1967 *46* 142—146

Moberger G B Lindstrom & L Andersson Freezedrying with a modified Glick Malmstrom apparatus *Exptl Cell Res* 1954 *6* 228—237

Mudge G H & I M Weiner The mechanism of action of mercurial and xanthine diuretics *Ann N Y Acad Sci* 1958 *71* 344—354

Murakami U Y Kameyama & T Kato Effects of vaginally applied contraceptive with phenylmercuric acetate upon developing embryos and their mother animals *Ann Rep Res Inst Environmental Med Nagoya Univ* 1956, 88—99

Nakajo S & K Tanaka Prolactin potency of the cephalic and the caudal lobe of the anterior pituitary in relation to broodiness in the domestic fowl *Poultry Sci* 1956 *35* 990—994

Nalbandov A R K Meyer & W H Mc Shan The role of a third gonadotropic hormone in the mechanism of androgen secretion in chicken testes *Anat Rec* 1951 *110* 475—493

Nordberg, G F & F Serenius Distribution of inorganic mercury in the guinea pig brain *Acta pharmacol et toxicol* 1969 *27* 269—283

Norén K & G Westoo Metylkvicksilver i fisk *Var foda* 1967 *2* 13—17 (In Swedish)

Ohmomo Y V Miettinen E Blankenstein & J K Miettinen Studies on the distribution of ^{203}Hg labelled methyl mercury and phenyl mercury in pike Fifth R. I S Symp 1969 Helsinki Finland

Palmer J S Mercurial fungicidal seed protectant toxic for sheep and chickens *J Am vet med Ass* 1963 *142* 1385—1387

Parry G & T Holliday An experimental analysis of the function of the pseudobranch in teleosts *J exp Biol* 1960 *37* 344—354

Payne F The cytology of the anterior pituitary of the fowl *Biol Bull* 1942 *82* 79—111

Pearse A G E Rapid freeze drying of biological tissues with a thermoelectric unit *J Sci Instr* 1963 *40* 176—177

Prickett C S E P Laug & F M Kunze Distribution of mercury in rats following oral and intravenous administration of mercury acetate and phenyl mercuric acetate *Proc Soc exp Biol* (N Y) 1950 *73* 585—588

D Iorio, A The release of catecholamines from the isolated chromaffine granules of the adrenal medulla using sulfhydryl inhibitors *Canad J Biochem Physiol* 1957, *35* 395—400

Jensen, S & A Jernelov Biosynthesis of mono and dimethylmercury *Nature* 1969, *223*, 1453—

Johnels, A G & T Westermark Studier på kvicksilverhalten hos några organismer i svensk natur Viltforskningsrådets nordiska konferens Foredrag och diskussioner, Statens veterinar medicinska anstalt, Stockholm 1967, 168—219 (In Swedish)

Johnels, A G, T Westermark, W Berg, P I Persson & B Sjostrand Pike (Esox lucius L) and some other aquatic organisms in Sweden as indicators of mercury contamination in the environment *Oikos* 1967, *18*, 323—333

Johnels, A G, M Olsson & T Westermark Esox lucius and some other organisms as indicators of mercury contamination in Swedish lakes and rivers *Bull Off int Epiz* 1968, *69*, 1439—1452

Kassel, B & E Brand The determination of methionine, cysteine and sulfate in proteins after hydrolysis with hydriodic acid *J Biol Chem* 1938, *125*, 145—159

King C V Mercury, its scientific history and its role in physical chemistry and electrochemistry *Ann N Y Acad Sci* 1957, *65*, 360—368

Kohn, J A lipoprotein staining method for zone electrophoresis *Nature* 1961, *189* 312—313

Kurland L T S N Faro & H Siedler Minamata disease *World Neurol* 1960, *1* 370—395

Laurent, P La pseudobranchie de Teleosteens, preuves electrophysiologiques de se fonctions chémoreceptrice et baroreceptrice *C r hebd Séanc Acad Sci Paris* 1967, *264* 1879—*1883*

Li, C H & H M Evans Chemistry of anterior pituitary hormones In The Hormones Acad Press, New York, 1948

Longsworth L G, R K Cannan & D A MacInnes An electrophoretic study of the proteins of egg white *J Am Chem Soc* 1940, *62* 2580—2590

Lundgren K D & Å Swensson Om fenylkvicksilverforeningar som yrkeshygieniskt problem *Nord Hyg Tidskr* 1950 *31* 207—219 (In Swedish)

Lush, I E The relationship of egg laying to changes in the plasma proteins of the domestic fowl *Brit Poult Sci* 1963 *4*, 255—260

Ludicke M Über eine rhytmische Ablagerung von radioaktivem Schwefel bei der Vogelfeder *Naturwiss* *1959, 23, 658—659*

Ludicke M Die Kennzeichnung der Zuwachsstreifen in der wachsenden Konturfeder mit Hilfe von radioaktivem Natriumsulfat *Z vergl Physiol* 1961 *44*, 133—173

Macfarlane E W E Effects of water source on toxicity of mercurial poisons *Growth* 1951 *15*, 241—266

Mc Indoe W M A lipophosphoprotein complex in hen plasma associated with yolk production *Biochem J* 1959 *72*, 153—159

Mc Kinley W M, W F Oliver W A *Maw & R H Common* Filter paper electrophoresis of serum proteins of the domestic fowl *Proc Soc Exptl Biol Med* 1953 *84* 346—351

Mann T The biochemistry of semen and of the male reproductive tract John Wiley and Sons New York 1954 1964

Marshall A J Biology and Comparative Physiology of Birds Acad Press, New York, 1960 1966

Tsuruga, H Tissue distribution of mercury orally given to fish *Bull Jap Soc Sci Fish* 1963 29 403—409

Ulfvarson U Distribution and excretion of some mercury compounds after long term exposure *Int Arch Gewerbepat Gewerbehyg* 1962 19 412—422

Ullberg S Studies on the distribution and fate of S[35] labelled benzylpenicillin in the body *Acta Radiol* 1954 *Suppl* 118 1—110

Ullberg, S Autoradiographic studies on the distribution of labelled drugs in the body *Second U N Int Conf Peaceful Uses of Atomic Energy* 1958 24 248—254

Vanstone W E W A Maw & R H Common Levels and partition of the fowl's serum proteins in relation to age and egg production *Canad J Biochem Physiol* 1955 33 891—903

Vogt G E Histochemical examination of the mercury occurring in the intestinal wall in mercury sublimate poisoning *Acta histochem* 1962 14 315—320

Wanntorp H K Borg, E Hanko & K Erne Mercury residues in wood pigeons in 1964 and 1966 *Nord Vet Med* 1967 19 474—477

Warkany J & D M Hubbard Acrodynia and mercury *J Pediat* 1953 42 365—386

Webb J L Enzyme and Metabolic Inhibitors Vol II Acad Press New York 1966

Weed L A & E E Ecker Phenyl mercuric compounds Their action on animals and their preservative values *J Inf Dis* 1933 52 354—363

Weiner I M K Garlid D Sapir & G H Mudge The effect of dimercaprol on the renal excretion of mercurials *J Pharmac exp Ther* 1959 127 325—331

Weiner I M R I Levy & H Mudge Studies on mercurial diuresis Renal excretion acid stability and structure-activity relationships of organic mercurials *J Pharmac exp Ther* 1962 138 96—112

Weissman N E B Schoenbach & E B Armistead The determination of sulfhydryl groups in serum *J Biol Chem* 1950 187 153—165

Westermark T Kvicksilver hos vattenlevande organismer In Kvicksilverfrågan i Sverige Stockholm 1965 25—76 (In Swedish)

Westöö G Kvicksilver i ägg *Vår föda* 1965 5 1—7 (In Swedish)

Westöö G Kvicksilver i ägg från svenska höns broiler och kycklingar samt i svensk kycklinglever *Vår Föda* 1966 a 7 85—92 (In Swedish)

Westöö G Methyl mercury compounds in fish identification and determination. *Acta Chem Scand* 1966 b 20 2131—2137

White I G The interaction between metals and chelating agents in mammalian spermatozoa *J exp Biol* 1956 33 422—430

Wilson W O U K Abbott & H Abplanalp Evaluation of Coturnix (Japanese Quail) as Pilot Animal for Poultry *Poultry Sci* 1961 40 651—657

Wingstrand K G The structure and development of the avian pituitary C W H Gleerup Lund Sweden 1951

Wood J M & F Kennedy Synthesis of methyl mercury compounds by extract of a methanogenic bacterium *Nature* 1968, 220 173—174

Yoshino Y T Mozai & K Nakao Distribution of mercury in the brain and its subcellular units in experimental organic mercury poisoning *J Neurochem* 1966 13 397—406

Zeyer H G Methoxyäthylquecksilberoxalatvergiftung *Zbl Arbeitsmed* 1952 2 68—72

Östlund K Studies on the metabolism of methyl mercury and dimethyl mercury in mice *Acta pharmacol et toxicol* 1969 27 suppl 1

Raeder M G & Snekvik Quecksilbergehalt mariner Organismen K Norske Vidensk Selsk Forh 1941 *13*, 169—172
Raeder M G & E Snekvik Mercury determinations in fish and other aquatic organisms K Norske Vidensk Selsk Forh 1949 a *21* 102—104
Raeder M G & E Snekvik Mercury contents of fish and other aquatic organisms K Norske Vidensk Selsk Forh 1949 b *21*, 105—109
Ramel C Genetic effects of organic mercury compounds *Hereditas* 1967 57 445—447
Rankama K & Th G Sahama Geochemistry Univ Chicago Press 1950
Romanoff, A L & A J Romanoff The Avian Egg John Wiley and Sons New York 1949
Romanoff A L The Avian Embryo The Macmillan Company New York 1960
Rothes G & H Havermann Versuche uber Verfutterungsmoglichkeit von mit Ceresan gebeiztem Getreide bei Huhnern Schweinen und Pferden *Nachr Schadlingsbekampfung* 1939 *14* 47—51
Rothstein A & A D Hayes The metabolism of mercury in the rat *J Pharm Exptl Therap* 1960 *130* 166—176
Schroeder H A Use of diuretic agents *J Am Med Assoc* 1951 *147* 1109—1118
Smart N A & M K Lloyd Mercury residues in eggs flesh and liver of hens fed on wheat treated with methyl mercury dicyandiamide *J Sci Fd Agric* 1963 *14* 734—740
Stock A & F Cucuel Die Verbreitung des Quecksilbers *Naturwiss* 1934 *22* 390—393
Stock A Der Quecksilbergehalt des menschlichen Organismus *Z Biochem* 1940 *304* 73—80
Sturkie P D Avian Physiology Cornell Univ Press 1954, 1965
Swensson Å Investigations on the toxicity of some organic mercury compounds which are used as seed disinfectants *Acta Med Scand* 1952 *143* 365—384
Swensson Å K D Lundgren & O Lindstrom Distribution and excretion of mercury compounds after single injection *Arch Industr Health* 1959 *20* 432—443
Swensson Å & U Ulfvarson Toxicology of organic mercury compounds used as fungicides *Occup Health Rev* 1963 *15* 5—11
Swensson Å & U Ulfvarson Distribution and excretion of various mercury compounds after single injections in poultry *Acta pharmacol et toxicol* 1968 a *3* 259—283
Swensson Å & U Ulfvarson Distribution and excretion of mercury compounds in rats over a long period after a single injection *Acta pharmacol et toxicol* 1968 b *26* 273—283
Takeda Y T Kunugi T Terao & T Ukita Mercury compounds in the blood of rats treated with ethylmercuric chloride *Toxicol Appl Pharmacol* 1968 *13* 165—173
Takeuchi T Pathology of Minamata disease In Minamata Disease Univ of Kumamoto Japan 1968
Tejning S & R Vesterberg Mercury in tissues and eggs from hens fed with grain containing methyl mercury dicyandiamide *Poultry Sci* 1964 *43* 6—11
Tejning S Biological effects of methyl mercury dicyandiamide treated grain in the domestic fowl Gallus gallus L *Oikos* 1967 a *Suppl* 8 pp 1—116
Tejning S Mercury in pheasants deriving from seed grain dressed with methyl and ethyl mercury compounds *Oikos* 1967 b *18* 334—344
Tillander M Utsondring av fenyl och metylmerkurinitrat efter oral administration eller intramuskular injektion i fisk mussla snacka och krafta *Nord Hyg Tidskr* 1969 *50* 181— 183 (In Swedish)
Timm F Zur Histochemie der Schwermetalle Das Sulfidsilberverfahren *Dtsch Z ges gerichtl Med* 1958 *46* 706—711
Tixier Vidal A B K Follett & D S Farner The anterior pituitary of the Japanese quail *Z fur Zellforsch und mikr Anat* 1968 *92* 610—635

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ACTA PHARMACOLOGICA
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VOLUMEN 27, SUPPLEMENTUM 2, 1969

ABSORPTION OF INJECTED INSULIN

A CLINICAL-PHARMACOLOGICAL STUDY

BY

CHRISTIAN BINDER



MUNKSGAARD COPENHAGEN 1969

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The experimental studies were performed at the Novo Research Institute, and my thanks are due to dr phil Jorgen Schlichtkrull, head of the institute, for many instructive discussions and much valuable criticism

For their inspiring collaboration, I thank Mr Klaus Jorgensen, cand polyt, and Mr Aage Volund, lic. tech, both from the Novo Research Institute

A prerequisite for the examinations was the readiness to co-operate shown by the patients In this connection I want to express my thanks to Mrs Edith Danielsen, matron at the Hvidore Hospital, for her active interest and patient collaboration at the clinical examinations

I am very grateful for the skillful technical assistance I received from the nurse Mrs Margrethe Nielsen, the laboratory technicians Mrs Jette Rasmussen, Mrs Inger Pless and Mrs Leila Klempel, and from the secretary Mrs. Becht

The figures were drawn by Mr Borge Clausen

It is a pleasure for me to acknowledge my indebtedness to all these persons and institutions as well as to others who have assisted me in completing this work

The thesis was completed in august 1968

Denne afhandling er af det lægevidenskabelige fakultet ved Københavns universitet antaget til offentligt at forsvares for den medicinske doktorgrad

København, den 9 september 1969

Dyre Trolle
h a dec

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statsaut translator

PRINTED IN DENMARK
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Contents

Contents

Introduction

Treatment of diabetes mellitus aims at attaining a metabolic balance and, if possible, aglucosuria and normal blood sugar concentration

In diabetic patients, metabolism can be controlled by regulating the diet and habits and by adjusting dose of insulin, time of injection, and type of insulin Due to differences in their methods of administration and courses of absorption, the various insulin preparations influence the blood sugar concentration in widely different ways.

Little is known of the conditions determining the absorption course, but estimates have been made by examining the effect of the absorbed insulin on the blood sugar concentration Based upon investigations performed under varying conditions in animals, normal persons, and patients with diabetes mellitus, a distinction has been made between insulin preparations having a quick, intermediate, slow or two-phase action Some of the investigations show, however, contradictory results Furthermore, the blood sugar concentration of the individual patient has often a complicated course that does not correspond to the theoretical schemes of the absorption and effect of the preparations Few direct examinations have been made of the absorption of insulin following subcutaneous or intramuscular injection

The aim of the present work was to find an effective method for determining the absorption of various pharmaceutical insulin preparations, and, in a large number of diabetic patients, to apply the method to examining the absorption following subcutaneous and intramuscular injections of therapeutic doses of these insulins Another aim was to elucidate the relation of the course of absorption to that of the blood sugar concentration

Chapter 1

METHOD FOR DETERMINING THE ABSORPTION OF INSULIN

By absorption of a substance is understood all the processes involved in transporting the substances from the site of application to the blood stream At any given time, the absorbed amount of a substance will be complementary to the non-absorbed amount, provided that the substance remains unchanged during the absorption, and that it does not disappear from the site of application in any way other than by absorption Under these conditions, the course of absorption can be determined by measuring either the amount of the substance conveyed to the vascular bed or the non-absorbed amount at the time in question

A determination of the amount of a substance conveyed to the blood is almost impracticable in clinical pharmacological studies, since it requires measurements of the blood flow through the tissue and of the arteriovenous concentration difference for the substance Determinations of the concentration in the blood will, however, give a measure of the course of absorption, provided that the distribution, decomposition, and excretion of the substance are known in detail

By labelling the substance with a γ-emitting isotope, the absorbed amount of the substance can be indirectly determined by external counting of the non-absorbed radioactivity, provided

1) that in every respect the labelled substance is absorbed like the unlabelled,
2) that neither the labelled nor the unlabelled substance are decomposed during the absorption, and
3) that the radioactivity counted externally is proportional to the non-absorbed amount of radioactivity

All examinations of the absorption of injected insulin have been performed by employing insulin labelled with radioactive iodine The course of absorption was determined by counting externally the remaining amount of radioactivity (Root, Irvine, Evans, Reiner and Carpenter 1944, Joiner

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1959 a and b, Moore, Mitchell and Chalmers 1959, Balodimos and Williams 1962, Nora, Smith and Cameron 1964, Binder, Nielsen and Jorgensen 1967, Dobson, Robbins, Johnsen, Mdalel, Odem, Cornwall and Davis 1967).

Reiner, Lang, Irvine, Peacock and Evans (1943) and Jorgensen and Binder (1966) alone have examined whether the conditions were fulfilled for employing iodine-labelled insulin to investigations of insulin absorption Using insulin-4-^{131}I-azobenzene, Reiner et al found in rabbit experiments that only minimal amounts of iodine were absorbed in the thyroid gland during the insulin absorption The authors took this finding to mean that little iodine was liberated during the absorption Previously, the same authors had shown that the iodinated and the non-iodinated preparation did not differ with regard to hypoglycemic effect (Lang and Reiner 1941) In 1966 Jorgensen and Binder published a summary of their results from a series of investigations of these problems The experimental method and the results will be given a detailed interpretation in this work

Insulins

The insulin employed was iodinated by mixing carrier free ^{125}I*) and ten times recrystallized insulin from the same species as that used for the pharmaceutical preparations The iodination technique was that described by Jorgensen (Jorgensen and Binder 1966). The degree of iodination was less than one atom of ^{124}I per 10 molecules of insulin The iodinated insulins were made from the same species as the non-iodinated ones The mixtures were thereafter treated as prescribed for the pharmaceutical preparations

*) During its disintegration to ^{125}Te ^{125}I emits γ rays (35 keV) and x rays (27 keV) The half-life is 60 days (Allen Smith and Hiscott 1961) The half thickness in tissue is 1.65 cm (Winter 1963)

Preparation	Species	State of the insulin	pH	Concentration of Zn^{++} (mg/100 ml)	Isotonica	Buffer	Abbreviation
Insulin Novo®	pig	solution	2 9	2 8	glucose	nil	SI
Actrapid®	pig	solution	7 4	0 6	sodium chloride	sodium acetate	A
Semilente®	pig	amorphous	7 4	8 8	sodium chloride	sodium acetate	SL
Ultralente®	beef	crystals	7 4	8 8	sodium chloride	sodium acetate	UL
C II	beef	crystals	7 4	1 3	sodium chloride	sodium acetate	C II

Table 1 1 Compositions of the non-compound insulins. All preparations contain 0 1% methyl para hydroxybenzoate

The compositions of the insulin preparations are given in tables I.I and I.II.

The specific radioactivity of the final preparations was approximately 0.4 μC/mg of insulin, corresponding to 0.6 μC/40 I.U. The specific radioactivities of the insulin components in each of the compound preparations agreed within ±5 per cent. The preparations were stored at 4°C.

The radiochemical stability was tested by examining for each preparation

Preparation	Species	State of the insulin	pH	Concentration of Zn^{++} (mg/100 ml)
Lente ®	pig[1] (30%) beef[2] (70%)	amorphous crystals	7.4	9
Rapitard ®	pig[3] (25%) beef[4] (75%)	solution crystals	7.0	1

1 identical with Semilente ®
2 identical with Ultralente ®
3 identical with Actrapid ®
4 identical with C II

Table I.II Compositions of the compound insulins. The preparations contain 0.1% methyl para hydroxybenzoate as a preservative. Isotonia was obtained by adding 0.7% sodium chloride. The buffer was 0.01 M sodium acetate.

to what extent the ^{125}I-labelled insulin followed the unlabelled insulin in repeated crystallization processes. Following determination of the total radioactivity, A, in a sample, so much unlabelled insulin, derived from the same species, was added that this amount, m, was at least 100 times greater than the insulin content in the sample.

After 4 crystallization processes, the specific radioactivity of the insulin, a, was determined. As m was made much larger than the amount of insulin in the sample taken, the amount of ^{125}I bound to insulin could be put approximately at $m \cdot a$. Hence, the relative insulin bound radioactivity (RIBR) was $\frac{m \cdot a}{A}$. Determinations of RIBR were performed at varying intervals and always immediately after the final application of a given preparation. RIBR was always found to exceed 0.95.

Determination of the absorption

The determinations of radioactivity were performed by use of a scintillation detector mounted with a 2"×2" NaI-crystal with a Be-window and coupled

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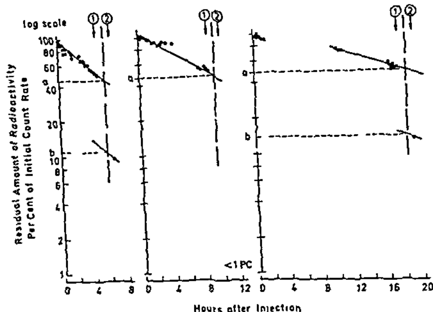


Figure 1.2 The amount of radioactivity measured externally in sheep after subcutaneous injection of Actrapid Semilente, and C II respectively At ①, local anesthesia was induced, and at ② the suturation after excision had been finished. Subsequently, one more measurement was made The excised amount of radioactivity was calculated by interpolating to the time halfway between ① and ② The values thus found are indicated on the ordinate axis by a and b

values given in table 1 III have been interpolated to the time halfway between these determinations, this is shown in figure 1 2

After being thawed, the tissue was homogenized in a mixture of ethanol and water containing 10 mg of bovine albumin (Armour, Cohn's fraction V) and acidified with hydrochloride acid to pH—2 The homogenate was filtrated on "Hyflo-Super Cel', and the filter cake was washed with acid aqueous ethanol The amounts of radioactivity were determined in filtrate, filter cake and one ml of the preparation injected into the tissue The filter cake contained from 3 to 10 per cent of the total amount of excised radioactivity The measurements were performed under comparable conditions Based thereupon, the amount of radioactivity in the excised tissue was calculated in percentages of the injected amount

The ethanol was removed under vacuum, and after filtration the proteins were precipitated with sodium chloride The precipitate was dissolved in diluted hydrochloride acid, and pH was adjusted to 7 4 After spinning,

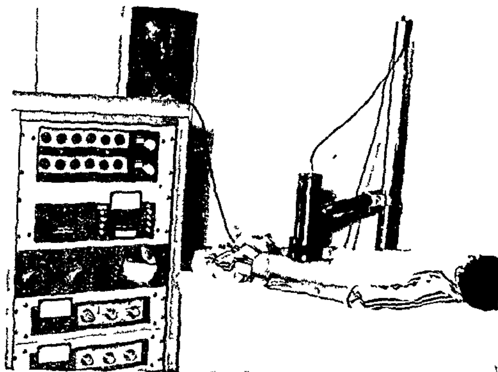


Figure 1 1 Arrangement of equipment for determination of absorption

to a spectrometer At a distance of one cm from the skin, the shielded detector was placed over the site of injection where the maximum counting rate was recorded (figure 1 1) The background activity counted contralateral to the site of injection, showed no significant increase in any of the examinations

Control examinations

Technique

An investigation of the reproducibility of the method was made in sheep For each of the non-compound insulins, one ml, corresponding to 40 i u, was injected subcutaneously into the gluteal region Immediately thereafter, the detector was placed over the site of injection, and the radioactivity was recorded, repeated registrations were made until the counts had fallen to about 50 per cent of their initial number At a good distance from the site of injection, local anesthesia was then induced with 2% Xylocaine® The tissue into which insulin had been injected was subsequently excised and frozen with dry-ice When the wound had been sutured, a final external counting was made of the remaining amount of radioactivity As there was approximately one hour between the last two external determinations, the

		SI	A	SL	CR	UL
RIBR	Preparation	98	97	96	100	99
(in per cent)	Extract	98	105	98	98	99

Table I IV The relative amounts of radioactivity bound to insulin (RIBR) in preparations and extracts The values denote in the individual sample the amount of radioactivity bound to insulin in percentages of the total amount.

the supernatant was an aqueous, purified extract of the tissue RIBR was determined in the preparations and in the alcoholic extracts alike (table I IV)

The insulin content was immunologically determined ad modum Heding (1966) *Insulin from the same species as the sample* was used as standard At least two sets of duplicate determinations were made, each set at different days At least two solutions were chosen in such a way that the determinations fell within the region of highest percentage accuracy

Finally, the concentrations of radioactivity (counts per minute per ml) were determined in preparations and extracts

The specific radioactivity (counts per milliunit (mU) of insulin) was then calculable

Experimental accuracy

The experimental error in the external determination originates partly in the counting rate partly in the counting geometry The counting rate varied between 1,000 and 4,000 c p m The coefficient of variation was between 1 6 and 3 2 per cent A measure of the error in the counting geometry was obtained by triplicate determinations The confidence limits stated in table I III were calculated on the basis of the above data

The error in determining the amount of radioactivity in the excised tissue consists in all essentials of the error in the counting rate, this was deduced from two examinations in sheep where, respectively 98 and 101 per cent of ^{125}I insulin were recovered after injection into excised tissue The coefficients of variation for the radioactivity measurements were most often less than one per cent and always less than 3 per cent

The relative insulin bound radioactivity was determined at duplicate measurements Based thereon, the standard deviation of the individual determination was found to be 0 9 per cent

When calculating the specific radioactivity, the error in the radioactivity measurements was expressed by the coefficients of variation, it never exceeded 0 9 per cent

Insulin preparation		SI	A	SL	C II	UL
Time of excision (hours after injection)		4 0	5 3	8 8	17 5	43 8
Radioactivity counted externally[1]) in percentages of initial counts	before excision	[2])	45	46	50	50
	after excision	[2])	11	0	15	1
Excised amount of radioactivity in percentages of initial counts	in vivo determinations	–	34 (27 8–40 2)[3])	46 (40 8–51 2)	35 (31 4–38 6)	49 (40 4–57 6)
	in vitro determinations	54	28	46	33	47

1) corrected values according to figures I 2 and I 3
2) no determinations
3) 95 per cent confidence limits

Table I III Results from counting externally, at the time of excision, the amount of radioactivity remaining at the site of injection compared with the results of determining the radioactivity in the excised tissue

		SI	A	SL	CH	UL
RIBR (in per cent)	Preparation	98	97	96	100	99
	Extract	98	105	98	98	99

Table 1.IV The relative amounts of radioactivity bound to insulin (RIBR) in preparations and extracts. The values denote in the individual sample the amount of radioactivity bound to insulin in percentages of the total amount

the supernatant was an aqueous, purified extract of the tissue. RIBR was determined in the preparations and in the alcoholic extracts alike (table 1 IV)

The insulin content was immunologically determined ad modum Heding (1966) Insulin from the same species as the sample was used as standard At least two sets of duplicate determinations were made, each set at different days At least two solutions were chosen in such a way that the determinations fell within the region of highest percentage accuracy

Finally, the concentrations of radioactivity (counts per minute per ml) were determined in preparations and extracts.

The specific radioactivity (counts per milliunit (mU) of insulin) was then calculable

Experimental accuracy

The experimental error in the external determination originates partly in the counting rate, partly in the counting geometry The counting rate varied between 1 000 and 4,000 c p m. The coefficient of variation was between 1 6 and 3 2 per cent A measure of the error in the counting geometry was obtained by triplicate determinations The confidence limits stated in table 1 III were calculated on the basis of the above data

The error in determining the amount of radioactivity in the excised tissue consists in all essentials of the error in the counting rate, this was deduced from two examinations in sheep where, respectively 98 and 101 per cent of ^{125}I insulin were recovered after injection into excised tissue The coefficients of variation for the radioactivity measurements were most often less than one per cent and always less than 3 per cent

The relative insulin bound radioactivity was determined at duplicate measurements Based thereon the standard deviation of the individual determination was found to be 0 9 per cent

When calculating the specific radioactivity, the error in the radioactivity measurements was expressed by the coefficients of variation, it never exceeded 0 9 per cent

Insulin preparation		SI	A	SL	C II	UL
Time of excision (hours after injection)		4 0	5 3	8 8	17 5	43 8
Radioactivity counted externally[1]) in percentages of initial counts	before excision	[2])	45	46	50	50
	after excision	[2])	11	0	15	1
Excised amount of radioactivity in percentages of initial counts	in vivo determinations	–	34 (27 8–40 2)[3])	46 (40 8–51 2)	35 (31 4–38 6)	49 (40 4–57 6)
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1) corrected values accord ng to figures 1 2 and 1 3
2) no determinations
3) 95 per cent conf dence l mits

Table I III Results from counting externally, at the time of excision the amount of radioactivity remaining at *the site of injection* compared with the results of determining the radioactivity in the excised tissue

Experiment	Concentration of radioactivity (cpm per ml)		Concentration of insulin (mU per ml)		Specific radioactivity (cpm per ml)		Spec. radioactivity (extract) / Spec. radioactivity (preparation)
	Preparation	Extract	Preparation	Extract	Preparation	Extract	
SI	37700 264[1])	352 3[1])	48143 7108[1])	419 53[1])	0 78	0 84	1 08
A	223280 417	866 16	43406 3333	210 15	5 14	4 13	0 80
SL	281265 281	2370 7	45025 2210	431 34	6 25	5 50	0 88
C II	281055 281	2892 11	51875 2559	570 63	5 42	5 07	0 94
UL	283500 284	3116 9	54763 9576	603 53	5 18	5 01	0 97
						Mean	0 93
						95 per cent confidence limits	0 80 – 1 06

1) SD of the single determination

Table I V The specific radioactivity of the various preparations and corresponding extracts

Multi-duplicate determinations were made of the insulin concentrations of the various solutions, and these determinations were repeated at *least* once for each solution. The coefficient of variation of the mean value ranged from 5 to 17 per cent. The individual standard deviations are listed in table 1.V.

Results

The results of the external measurements are given in the figures 1.2 and 1.3. Due to technical errors, the external measurements could not be completed as far as the acid insulin solution was concerned. For the other preparations, it took 5.3 to 43.8 hours for the amount of radioactivity to fall to approximately 50 per cent of the initial amount. The insulin for which the radioactivity declined most rapidly was A (5.3 hrs.), then followed SL (8.8 hrs.), C II (17.5 hrs.), and finally UL (43.8 hrs.). The duration of the excision was about one hour including induction of local anesthesia (marked ① in the figures 1.2 and 1.3) and suturation of the wound (② in figures 1.2

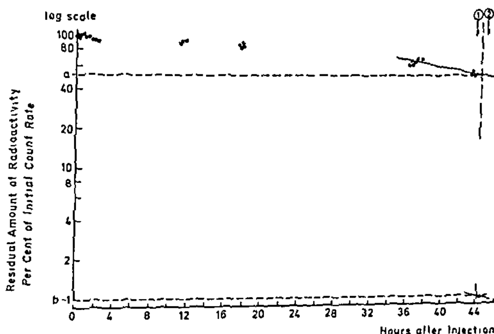


Figure 1.3. The amount of radioactivity measured externally in sheep after subcutaneous injection of Ultralente. At ①, local anesthesia was induced, and at ②, the suturation after the excision had been finished. Subsequently, one more measurement was made. The excised amount of radioactivity was calculated by interpolating to the time halfway between ① and ②. The values thus found are indicated on the ordinate axis by a and b

Experiment	Concentration of radioactivity (cpm per ml)		Concentration of insulin (mU per ml)		Specific radioactivity (cpm per ml)		Spec. radioactivity (extract) / Spec. radioactivity (preparation)
	Preparation	Extract	Preparation	Extract	Preparation	Extract	
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and 1 3) The radioactivity was counted immediately before and after excision The local anesthetic did not contain epinephrine or nor-epinephrine So it must be assumed that the absorption continued as long as a circulation remained in the injected tissue By interpolating the values measured externally at the induction of anesthesia and immediately after the suturation, the radioactivity at the time halfway between these two measurements was assessed The interpolation was made on the semilogarithmic graphs of the results shown in figures 1 2 and 3 With regard to C H and UL, one line representing the fall in radioactivity at the site of injection, could not reasonably be drawn In these two cases, the line was drawn corresponding to the last two sets of measurements before induction of local anesthesia The values found by interpolation are indicated on the ordinate axis by a and b Table 1 III contains also the results from *in vitro* measurements of the amount of radioactivity in the excised tissue The values are given in percentages of the injected amounts Taking into account the above-mentioned errors, the amount of excised radioactivity calculated from the external measurements did not in any case differ significantly from the amount found in the tissue *in vitro*

Table 1 IV demonstrates that practically all the radioactivity was bound to insulin in the preparations and the purified extracts alike

The values for specific radioactivity of the aqueous extracts and of the corresponding preparations are given in table 1 V In the case of the acid insulin solution, the specific radioactivity exceeded that of the preparation by 8 per cent In the other four preparations, the specific radioactivity of the extract was lower than that of the preparation This was most pronounced as for the neutral and the amorphous preparation, where the specific radioactivities were, respectively, 20 and 12 per cent lower than in the extracts Mean of ratio between connected values of specific radioactivity was 0 93 This value is not significantly different from one

Discussion

The reproducibility of the counting geometry decides whether the ratio of the counts to the residual amount of radioactivity is constant during the absorption As the detector could not be fixed over the site of injection for the total duration of the absorption, it was at each measurement placed where the maximum counting rate was found Consequently, it must be assumed that the detector was centred in relation to the radioactivity at each counting The half thickness for ^{125}I in tissue being only 1 7 cm means

that an alteration of the distance between a given amount of radioactivity and the surface will change the number of counts recorded The counting geometry of a radioactive molecule will be less optimal if, during the absorption, the molecule is removed from the centre of the radioactivity space in a direction parallel to or pointing away from the terminal surface of the detector Such changes in the distribution of radioactivity may take place in the first minutes after the injection where the solvent is supposed to disappear (Hewitt 1954) Diffusion in the tissue, too, may change the distribution and thereby the *counting geometry* If some of the injected amount *is absorbed more rapidly* than the rest, this will alter the relation between the actual residual amount of radioactivity in the tissue and that calculated from the external measurement

The results from the excision experiments showed, however, that the amount of radioactivity in the excised tissue did not differ significantly from that calculable from the external measurements, provided that the excised amount made up 45 per cent or more of the injected amount As the said possible changes in the counting geometry must have at least the same effect during the first part of the absorption as during the last part, there are no grounds for assuming that the ratio of counts to the residual amount of radioactivity should not be constant during the whole of the absorption

The second part of the experiment demonstrated that practically all the radioactivity was bound to insulin and that it did not exist in the form of iodide or iodinated breakdown products

The results from determining the specific radioactivity in preparations and corresponding extracts were decisive of the applicability of the method In three sets of five (SI, C II, and UL), the difference between the specific radioactivity of the preparation and of the extract was less than 10 per cent In the case of Actrapid and Semilente, the specific radioactivities of the extracts were, respectively, 20 and 12 per cent lower than of the preparations Identical procedures were employed for making the iodinated insulins of the individual preparations The two preparations A and SL had no common factor to distinguish them from the others As all determinations were performed in the same way, the four determinations can be considered collectively when calculating the total experimental *error in the determination* of specific radioactivity Mean of ratio between connected values of specific radioactivity was 0 93, 95 per cent confidence limits 0 80–1 06 The ratio of labelled to unlabelled insulin, thus, can have changed but little during the stay in the tissue This means that labelled insulin acts like unlabelled during the absorption process

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The unchanged specific radioactivity shows that the labelled insulin is absorbed in the same way as is the unlabelled (point 1, page 11) Together with the finding that the total amount of radioactivity was bound to insulin, this gives grounds for asserting that insulin was not decomposed during the absorption Had it been, a lower RIBR in the tissue was to be expected due to breakdown products of iodinated insulin (point 2, page 11) Proportionality was, moreover, demonstrated between the amount of externally counted radioactivity and the non-absorbed amount (point 3, page 11)

Thus, there is basis for assuming that external counting over a subcutaneous depot of ^{125}I-labelled insulin provides a reliable measure of the insulin absorption

Chapter 2

MECHANISM OF INSULIN ABSORPTION

As mentioned in chapter one, few investigations have been made of insulin absorption following subcutaneous or intramuscular injection None of the investigations include examination of the mechanism of absorption

Beecher and Krogh (1936) demonstrated that precipitated insulin was conveyed into the small lymph capillaries where it apparently was dissolved Besides being absorbable via the lymphatic system, the injected insulin *might possibly* be absorbed directly from tissue to blood No data are reported on this route of absorption

Employing insulin solutions in therapeutic doses, Binder, Nielsen and Jorgensen (1967) investigated the absorption following subcutaneous injection Irrespective of the region of injection, the absorption was characterized by a *relative* absorption rate (figure 2 1) that increased during the first hours after the injection. The relative absorption rate denotes the ratio of the amount absorbed per unit of time to the residual amount. The terminal part of the curve showed a mono-exponential fall corresponding to a constant relative absorption rate The relative absorption rate following intramuscular injection was likewise found to have this initial increase (see chapter 3)

According to Moore, Mitchell and Chalmers (1959) the initial increase in the relative absorption rate took place only after injection of highly concentrated insulin solutions whereas the absorption rate was constant in the case of low concentrations

Joiner (1959 a and b) found the insulin absorption to have a mono-exponential course after a subcutaneous injection of therapeutic doses In his investigations variations in insulin concentration and injection volume did not have any demonstrable influence on the course of the absorption

Prior to advancing a theory for the absorption of insulin solutions, the following problems had to be examined experimentally

1 Is there at all a direct absorption of insulin to the blood within the injected tissue

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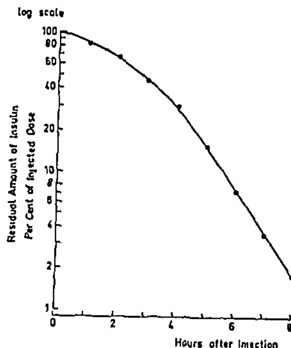


Figure 2 1 Absorption curve following a single subcutaneous injection of 0 3 ml Actrapid into the femoral region

2 Is the blood flow through the injected tissue correlated to the absorption of insulin

3 Is the absorption systematically correlated to injection volume and insulin concentration

Experimental examinations

1 Direct absorption from tissue to blood

The examinations were performed with unlabelled insulins of the following types Actrapid, Semilente, and C II, a suspension of beef insulin crystals The compositions of the preparations are given in table 1 1

The examinations were made in patients without symptoms of diabetes mellitus None had been treated with insulin before During arrest of venous return from the forearm, the site of injection was chosen so that the venous outflow was clearly depicted With the needle distal to the cubital fossa, a catheter was placed in the vein draining the chosen region Another catheter was placed in any contralateral arm vein

After blood sampling for determination of the spontaneous insulin concentration, a subcutaneous injection of insulin was given At varying intervals, blood was sampled for determination of the insulin concentration

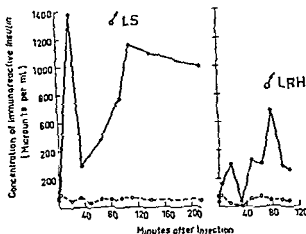


Figure 2.2 Concentration of immunoreactive insulin in blood from an arm vein following subcutaneous injection of 0 3 ml of Actrapid into the same arm (unbroken curve) Control determinations were made in venous blood from the contralateral arm (dashed curve)

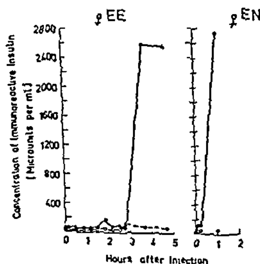


Figure 2.3 Concentration of immunoreactive insulin in blood from an arm vein following subcutaneous injection of 0.5 ml of Semilente into the same arm (unbroken curve) Control determinations were made in venous blood from the contralateral arm (dashed curve)

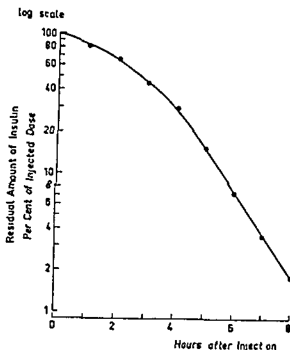


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After blood sampling for determination of the spontaneous insulin concentration, a subcutaneous injection of insulin was given At varying intervals, blood was sampled for determination of the insulin concentration

than that found simultaneously in venous blood from a corresponding tissue region into which no insulin had been injected.

It must be assumed that there is no route from the lymphatic system to the blood distal to the cubital fossa, where the first lymphatic node of the arm is situated. The results, consequently, can mean nothing but insulin bieng absorbed directly from tissue to blood.

As the blood flow was not measured, and as the total amount of blood in the injection region not with certainty was drained into the vein from which the sample was taken, no conclusion can be drawn about the amount of injected insulin absorbed in this way.

2. Insulin absorption and blood flow

The courses of insulin absorption from the anterior tibial muscle and other skeletal muscles did not differ from that following subcutaneous injection (see chapter 3). As there existed an established method for determining the blood flow through the anterior tibial muscle by use of ^{133}Xe, this muscle was chosen for examination of the correlation between insulin absorption and blood flow through the injected tissue.

A dose of 0.3 ml (40 i. u. per ml) of Insulin Novo (SI in table 1.I) was employed for the determination. The site of injection in the anterior tibial muscle was 10 cm distal to the lower edge of patella and 1 to 2 cm lateral to the anterior border of tibia. The injection was made at a depth of about 2 cm. For 15 minutes before the examination, the patient had to lie in a supine position with the leg to be examined fixed and the knee half bended. The residual amount of insulin was counted externally every 15 minutes for the first two hours after the injection, then every hour or half hour for the next six hours.

The muscle blood flow (MBF) was determined by use of ^{133}Xe injected into the same place as the insulin. The procedure followed exactly that described by Munck, Lindbjerg, Binder, Lassen, and Trap-Jensen (1966).

The principles of calculation of MBF and their theoretical basis were described by Lassen, Lindbjerg, and Munck (1964). From a semilogarithmic curve of the ^{133}Xe absorption, MBF is calculable as

$$MBF = \lambda \frac{0.693}{t_{1/2}} \text{ml per 100 g tissue per min} \qquad (1)$$

where λ is the partition coefficient between tissue and blood. According to Conn (1961), $\lambda_{Xe} = 0.73$, $t_{1/2}$ is the half time for the mono-exponential absorption curve for ^{133}Xe.

Determinations were made of MBF in the resting muscle, and of the peak

The concentration of insulin was immunologically determined a m Heding (1966)

During the first 20 minutes after injection of Actrapid, an increase was seen in the insulin concentration in the blood from the arm concerned (figure 2 2) After a fall, the concentration rose again to reach its peak value approximately 90 minutes after the injection

Following injection of Semilente, 3 and a half hours elapsed in one case (EE) before the insulin concentration differed significantly in the blood samples taken simultaneously from both arms (figure 2 3) In the other case (EN), a similar difference was found as early as one hour after the injection This patient developed pronounced hypoglycemic symptoms, which disappeared a few minutes after subcutaneous injection of glucagon

Figure 2 4 shows that 30 to 60 minutes after an injection of suspended beef insulin crystals (CII) an increase was demonstrable in the insulin concentration of the venous blood from the arm concerned The last blood samples were taken about 24 hours after the injection They showed the insulin concentration still to be considerably higher in the venous blood from the arm where the injection had been given

There was never any demonstrable increase in the insulin concentration in venous blood from the control arm

The investigation showed that, irrespective of whether the insulin was injected in a dissolved, amorphous or crystalline state, the insulin concentration in venous blood from the region of injection was considerably higher

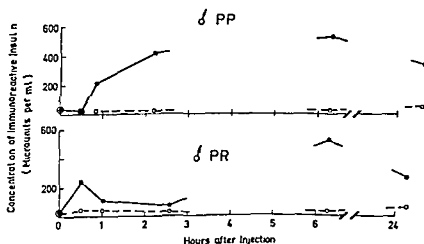


Figure 2 4 Concentration of immunoreactive insulin in venous blood from an arm following subcutaneous injection of 0 5 ml of C II into the same arm (unbroken curve) Control determinations were made in venous blood from the contralateral arm (dashed curve)

than that found simultaneously in venous blood from a corresponding tissue region into which no insulin had been injected

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Following injection of Semilente, 3 and a half hours elapsed in one case (EE) before *the insulin* concentration *differed significantly* in the blood samples taken simultaneously from both arms (figure 2 3) *In the other* case (EN), a similar difference was *found* as early as one hour after the injection This patient developed *pronounced* hypoglycemic symptoms, which disappeared a few minutes after *subcutaneous* injection of glucagon

Figure 2 4 shows that 30 to 60 minutes after an injection of suspended beef insulin crystals (*CII*) an increase was demonstrable in the insulin concentration of the venous blood *from the* arm concerned The last blood samples were taken about *24 hours after* the injection They showed the insulin concentration *still to be considerably* higher in the venous blood from the arm where the *injection* had been given

There was never any demonstrable increase in the insulin concentration in venous blood from the control arm

The investigation showed that, *irrespective* of whether the insulin was injected in a dissolved, amorphous or *crystalline state*, the insulin concentration in venous blood *from the region* of injection was considerably higher

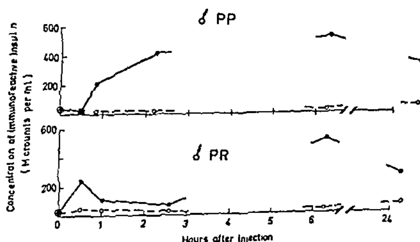


Figure 2 4 Concentration of immunoreactive insulin in venous blood from an arm following subcutaneous injection of 0 5 ml of C II into the same arm (unbroken curve) Control determinations were made in venous blood from the contralateral arm (dashed curve)

than that found simultaneously in venous blood from a corresponding tissue region into which no insulin had been injected

It must be assumed that there is no route from the lymphatic system to the blood distal to the cubital fossa, where the first lymphatic node of the arm is situated The results, consequently, can mean nothing but insulin bieng absorbed directly from tissue to blood

As the blood flow was not measured, and as the total amount of blood in the injection region not with certainty was drained into the vein from which the sample was taken, no conclusion can be drawn about the amount of *injected insulin* absorbed in this way

2 Insulin absorption and blood flow

The courses of insulin absorption from the anterior tibial muscle and other skeletal muscles did not differ from that following subcutaneous injection (see chapter 3) As there existed an established method for determining the blood flow through the anterior tibial muscle by use of ^{133}Xe, this muscle *was chosen for examination of the correlation between insulin absorption* and blood flow through the injected tissue

A dose of 0 3 ml (40 i u per ml) of Insulin Novo (SI in table 1 I) was employed for the determination The site of injection in the anterior tibial muscle was 10 cm distal to the lower edge of patella and 1 to 2 cm lateral to the anterior border of tibia The injection was made at a depth of about 2 cm For 15 minutes before the examination, the patient had to lie in a supine position with the leg to be examined fixed and the knee half bended The residual amount of insulin was counted externally every 15 *minutes for* the first two hours after the injection, then every hour or half-hour for the next six hours

The muscle blood flow (MBF) was determined by use of ^{133}Xe injected *into the same* place as the insulin The procedure followed exactly that described by Munck, Lindbjerg, Binder, Lassen, and Trap-Jensen (1966)

The principles of calculation of MBF and their theoretical basis were described by Lassen, Lindbjerg, and Munck (1964) From a semilogarithmic curve of the ^{133}Xe absorption, MBF is calculable as

$$\text{MBF} = \lambda \frac{0\ 693}{t_{1/2}} \text{ml per 100 g tissue per min} \qquad (1)$$

where λ is the partition coefficient between tissue and blood According to Conn (1961), $\lambda_{Xe} = 0\ 73$, $t_{1/2}$ is the half time for the mono-exponential absorption curve for ^{133}Xe

Determinations were made of MBF in the resting muscle, and *of the* peak

flow, when the patients had performed 80 to 1,000 dorsiflections of the foot during 2 to 5 minutes of arterial ischemia

At an interval of 1 to 2 days, the two examinations were performed in 9 diabetic patients without signs of arterial disturbances in the legs All patients had been treated with insulin during at least one year.

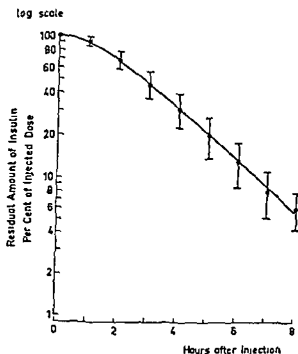


Figure 2 5 Average curve of the absorption following injection of 0 3 ml of Insulin Novo into the anterior tibial muscle Confidence limits 95 per cent

Figure 2 5 shows a curve of the average insulin absorption, confidence limits 95 per cent The shape of the curve is similar to that shown in figure 2 1 The rate constants, k, during the first four 30-minute periods and during the terminal, mono-exponential part were calculated for each absorption curve by applying the following formula

$$k = \frac{\log C_1 - \log C_2}{0\ 434\ (T_2 - T_1)} \qquad (2)$$

where C_1 and C_2 are the residual amounts of insulin at the times T_1 and T_2

The results are given in table 2 I The rate constants increased significantly from the first to the third and the subsequent 30-minute periods and from the second 30-minute period to the terminal one (120 – 480 min)

Table 2 I also contains the MBF values found with the ^{133}Xe technique

Case No.	MBF (ml min⁻¹ per 100 g)		Insulin, rate constants (min⁻¹ · 10⁻²)				
	peak flow	resting	0–30	30–60	60–90	90–120	120–480
363	47	2.6	0	0.21	0.38	0.51	0.83
364	79	2.2	0.10	0.04	0.29	0.32	0.94
365	24	1.6	0.03	0.07	0.36	0.41	0.61
366	97	0.8	0.03	0.10	0.22	0.35	0.70
370	60	2.6	0.10	0.44	0.65	0.69	0.75
371	66	1.6	0	0.46	0.63	0.69	0.70
372	45	5.6	0.39	0.66	1.07	1.38	1.61
373	32	2.0	0	0.10	0.29	0.36	0.67
375	48	3.2	0.21	0.38	0.33	0.37	0.96
Mean	50	2.5	0.10	0.27	0.47	0.56	0.86
95 per cent confidence limits	27-83	1.4-3.5	0-0.20	0.11-0.44	0.26-0.68	0.30-0.82	0.60-1.13
Coefficient of correlation for resting MBF *versus insulin rate constants*			0.842	0.735	0.777	0.940	0.868
P for the correlation			<0.001	<0.01	<0.005	<0.001	<0.001

Table 2.I The blood flow in the anterior tibial muscle correlated to the rate constants for insulin absorption following injection of an insulin solution (SI) into the same muscle

A highly significant correlation was demonstrated between insulin absorption and resting muscle blood flow

The insulin absorption and the blood flow were determined at two different times so the insulin absorption can not have influenced the blood flow measured

This fact together with insulin being directly absorbable from tissue to blood seems to indicate that an essential part of the absorption is direct Besides ascertaining that all samples are taken from a vein containing the total amount of blood from the region of injection, a conclusive, experimental proof demands determination of the velocity of flow in this vein

3 *Injection volume and insulin concentration*

For practical reasons no more than three determinations of absorption could be carried out simultaneously in the individual patient The investigation was therefore planned as a 3 × 3 latin square examination with 3 different volumes and 3 different concentrations (*table 2 II*)

The investigation comprising two identical series a total of 18 absorption determinations was made in 6 patients All patients had been insulin

treated for at least one year. None suffered from lipodystrophy, or had any other symptoms of skin disease. A subcutaneous injection was given proximally in either femoral region. A third injection was given 15 cm distal to one of the proximal injections. All examinations started at 8 a.m. The radio-

conc \ volume	1 ml	0 1 ml	0 001 ml
40 i u /ml	x	z	y
4 i u /ml	y	x	z
0 4 i u /ml	z	y	x

Table 2 II Programme for examining the effect of variations in volume and concentration For each series 3 patients, x, y, and z were examined

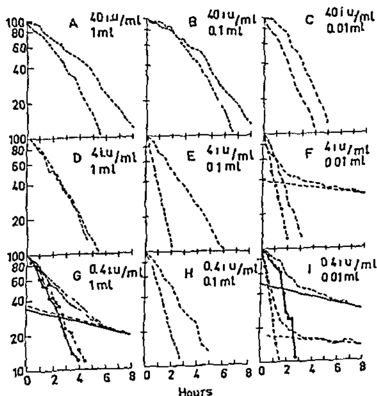


Figure 2 6 Absorption curves of insulin in a neutral solution injected in various volumes and concentrations The investigation was carried out according to the programme shown in table 2 II A subtraction was made of the terminal part of the curve where this was slower The initial course is shown by the unbroken curve

activity was counted as described on page 13 Registrations were made every 15 minutes during the first 3 hours after the injection, thereafter every 30 minutes for 5 hours

As tracer was used ^{125}I insulin in Actrapid medium. The injected amount of radioactivity was approximately 0 1μC per injection

The results are given on a semilogarithmic scale in figure 2 6 The residual amount of insulin showed a simple mono-exponential fall after injection of 0 1 ml in concentrations of 4 and 0 4 i u per ml (figure 2 6, E and H) At the same concentrations, the initial absorption rate was slower when the volume was increased ten times (figure 2 6, D)

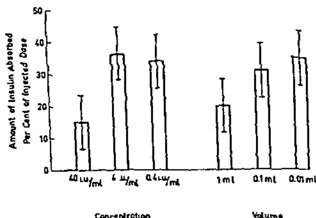


Figure 2 7 The mean relative amounts of insulin absorbed during the first hour after the injection arranged according to volume and concentration. Confidence limits 95 per cent.

This finding was even more pronounced when the concentration was 40 i u /ml (figure 2 6, A, B and C) The other absorption curves (figure 2 6, F G and I) had a two-phase course with a rapid initial phase followed by a slower component

Figure 2 7 shows the average amounts of insulin absorbed during the first hour after the injection calculated for three different volumes and for three different concentrations, confidence limits 95 per cent At a concentration of 40 i u /ml, the absorbed amount was significantly smaller than at concentrations of 4 and 0 4 i u /ml The amount of insulin absorbed during the first hour showed a tendency to increase with decreasing volume

treated for at least one year. None suffered from lipodystrophy, or had any other symptoms of skin disease. A subcutaneous injection was given proximally in either femoral region. A third injection was given 15 cm distal to one of the proximal injections. All examinations started at 8 a.m. The radio-

conc. \ volume	1 ml	0 1 ml	0 001 ml
40 i u /ml	x	z	y
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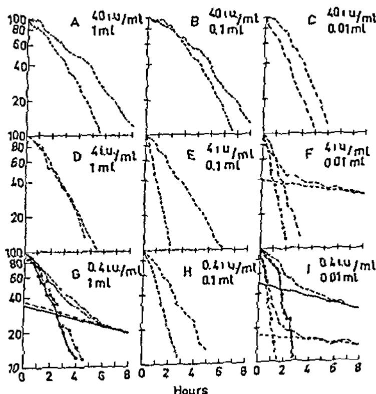


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an initial depression of the absorption at insulin concentrations of 40 i u / ml and likewise a depressing influence when the injection volume was increased from 0 01 ml to 1 ml

A similar correlation between injection volume and absorption was previously demonstrated by Warner, Dobson, Pale, Johnston, and Finney (1953) who found the relative absorption rate of intramuscularly injected ²⁴Na to fall with increasing injection volume Sund and Schou (1964 a) found that the relative absorption rates of intramuscularly injected mannitol and sucrose decreased with increasing injection volume

Himwick, Goldman, and Krosnick (1932) demonstrated that following a subcutaneous injection of glucose, the relative absorption rate was smaller in the case of a 50 per cent solution than in the case of 2 5 and 5 per cent solutions Sund and Schou found no demonstrable effect by varying the concentrations of sucrose

Sund and Schou (1964 b) showed, on the other hand, that the relative absorption rate of atropine injected intramuscularly in concentrations above 0 5 mg/ml fell with increasing concentration Simultaneously injected sucrose was likewise depressed Their findings confirmed Schnitman and Kondritzer s (1957) observation that the absorption curve for atropine changed from being mono exponential at small atropine concentrations to showing an increasing relative absorption rate during the first 30 minutes at higher concentrations

Sund and Schou advanced various possible explanations of the atropine absorption and the concomitant depression of the sucrose absorption As shown by Oroszlan and Maengwyn Davies (1962), atropine reacts with proteins and amino acids A possible explanation is that atropine triggered a reduction of the capillary membrane area and/or a reduction of the velocity of flow in the absorbing capillaries

Altura (1966) has later shown that local application of atropine caused contraction of arterioles metarterioles and precapillaries The dosage regimen employed had a specific counter action on the vasodilating effect of acetylcholine A reduced perfusion thus seems to be the explanation of atropine depressing its own absorption and that of sucrose

A great number of substances including some polypeptides, influence the microcirculation when applied locally Thus, having an arteriolar contracting effect angiotensin induces ischemia (Litton Walter, and Berman 1966) Applied in small doses bradykinin results in an increased blood flow followed by reactive ischemia Larger doses may induce stasis or exudation (Hyman and Paldino 1966) Vasopressin causes vasoconstriction and ischemia (Altura and Zweifach 1966)

Source of variation	Degrees of freedom	s^2	F ratio	P value
Between patients	5	446.6	5.81	< 0.05
Between conc.s	2	797.5	10.37	< 0.01
Between volumes	2	338.5	4.40	≈ 0.05
Residual	8	76.9	–	–

Table 2 III Analysis of variance of the residual amounts of insulin one hour after the injection of insulin in different volumes and concentrations in the different patients according to the programme given in table 2 II

Table 2 III shows the result of an analysis of variance made on the residual amounts of insulin measured one hour after the injection.

The residual amount of insulin differed significantly between patients ($p < 0.05$) and between concentrations ($p < 0.01$). P-value of difference between volumes was 0.05.

Thus, the results showed that injection volume and insulin concentration are of importance for the absorption. The absorption rate decreased with increasing concentration as well as with increasing volume. The greatest effect of a given variation was found when the concentration was altered.

Discussion

Initial part of the absorption

The subcutaneous absorption was studied in detail by Schou (1959 and 1961). He demonstrated in rats that the subcutaneous absorption of drugs is influenced by a self-depression, whereby is understood a delay of the absorption caused by endogenously liberated compounds. The absorption of subcutaneously injected substances was furthered by simultaneously injecting an antihistaminic agent as well as by depleting the animals of histamine by precedent application of compound 48/80, a histamine liberator. Local injection of histamine, on the other hand, depressed the absorption. Consequently, it was assumed that histamine had a major influence on the self depression of subcutaneous absorption in rats. It was emphasized that no conclusion could be drawn on these examinations with regard to a similar mechanism being involved in the subcutaneous absorption in other species. As potential factors triggering off the self-depression, Schou states the injected substance, the composition of the injected fluid, including its pH, the injection trauma, and the injection pressure alike.

The present investigations of the absorption of insulin solutions showed

an initial depression of the absorption at insulin concentrations of 40 i u / ml and likewise a depressing influence when the injection volume was increased from 0 01 ml to 1 ml

A similar correlation between injection volume and absorption was previously demonstrated by Warner, Dobson, Pale, Johnston, and Finney (1953) who found the relative absorption rate of intramuscularly injected ^{24}Na to fall with increasing injection volume Sund and Schou (1964 a) found that the relative absorption rates of intramuscularly injected mannitol and sucrose decreased with increasing injection volume

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It seems reasonable to assume that local application of insulin in pharmacological concentrations has a direct effect on the microcirculation in the region concerned. If so, the effect consists in a vasoconstriction causing a reduction of the capillary membrane area and consequently a depressed absorption. The effect will moderate, partly due to a fall in insulin concentration, partly due to active attempts by the tissue to counteract the resulting 'ischemia'.

The correlation found between injection volume and insulin absorption, where increasing volume depressed the absorption, might, however, be a concealed concentration effect. If, at increasing volumes, the injected insulin does not spread to a correspondingly larger tissue region, the insulin concentration in the tissue will likewise increase – and the increment will be of such a size that it influences the absorption to a measurable degree.

Another possible explanation of the increasing relative absorption rate is that the zinc ions of the insulin molecule are gradually liberated, whereby a dissociation to smaller insulin molecules takes place (Marcker 1960 a and b).

Figure 2.5 shows the result from injecting subcutaneously 0.3 ml (12 i.u.) of two crystalline pig insulin solutions – one identical with Actrapid and containing 6 μg of Zn^{++} per ml, the other containing less than 0.3 μg of

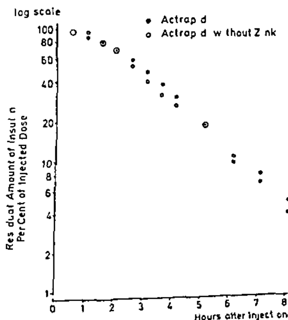


Figure 2.8 Absorption curve from one patient following subcutaneous injections of 0.3 ml of Actrapid with the prescribed amount of Zn^{++} and without Zn^{++}

Zn^{++} per ml, but otherwise composed like Actrapid There was no difference between the two courses of absorption, neither with regard to the initial depression of the absorption, nor with regard to the mono-exponential part Neither was there any demonstrable difference between absorption of *SI* and of an acid solution of crystalline pig insulin containing 40 i u of insulin per ml and less than 0 3 μg of Zn^{++} per ml

Thus, the zinc content of the insulin solution is of no importance for the absorption

Mono-exponential part of the absorption curve

The present investigations gave basis for the assumption that the absorption of dissolved insulin is closely correlated to the blood flow through the tissue

Let it be assumed that the vein draining the site of injection in the examinations mentioned on page 24, drains a total of 400 g of tissue, the blood flow of which is 8 ml per 100 g of tissue per min (Holzman, Wagner, Iio, Rabinowitz, and Zierler 1964), this corresponds to 1920 ml per 400 g of tissue per hour The venous insulin concentration of LS in figure 2 2 was 1400×10^{-6} units per ml during the period 90–150 minutes after the injection Thus, during this interval 1 92 $\times$ 1 4 units = 2 7 units were removed per hour There are no data on the insulin absorption rate following injection into the arm. However, according to Munck, Andersen, and Binder (1967) the blood flow in the subcutaneous tissue of the arm equals that in the abdominal subcutis It appears from figure 3 3 that approximately 25 per cent of Actrapid injected into the latter region are absorbed during the interval 90 minutes to 150 minutes after the injection The absorbed amount is in this case 3 units

Though the calculation is to some extent based on an estimate, the result indicates that a considerable part of the injected insulin is absorbed directly to the blood

Investigations by Ismail, El Ride, Abdel Hay, Kamel, Talaat, and Fayek (1967) with ^{131}I insulin injected intramuscularly suggest that some of the insulin absorption takes place via the lymphatic system They employed an insulin preparation dissolved in glycine NaOH buffer at a pH of 9 3 and having a very high specific radioactivity (566μC/mg) The preparation examined therefore, is not identical with those used in the present work The results seem moreover to indicate that a considerable decomposition has taken place during the absorption of the iodine insulin So, based upon their results no conclusions can be drawn with certainty about the therapeutic conditions

Factors determining the absorption from tissue to blood of a substance,

It seems reasonable to assume that local application of insulin in pharmacological concentrations has a direct effect on the microcirculation in the region concerned If so, the effect consists in a vasoconstriction causing a reduction of the capillary membrane area and consequently a depressed absorption The effect will moderate, partly due to a fall in insulin concentration, partly due to active attempts by the tissue to counteract the resulting 'ischemia'

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Another possible explanation of the increasing relative absorption rate is that the zinc ions of the insulin molecule are gradually liberated, whereby a dissociation to smaller insulin molecules takes place (Marcker 1960 a and b)

Figure 2 5 shows the result from injecting subcutaneously 0 3 ml (12 i u) of two crystalline pig insulin solutions – one identical with Actrapid and containing 6 μg of Zn^{++} per ml, the other containing less than 0 3 μg of

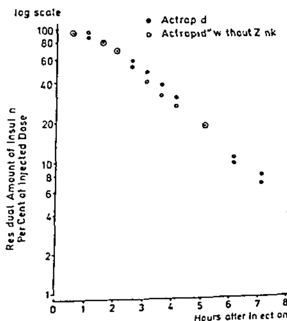


Figure 2 8 Absorption curve from one patient following subcutaneous injections of 0 3 ml of Actrapid with the prescribed amount of Zn^{++} and without Zn^{++}

Chapter 3

ABSORPTION OF DISSOLVED INSULIN

By dissolved insulin is understood a solution of crystalline insulin to which no agents are added in order to prolong the absorption or action of the insulin

Subcutaneous injections

Moore, Mitchell and Chalmers (1959) employed a neutral insulin solution in small concentrations (less than 0 4 i u per ml) for subcutaneous injections into the deltoid region They demonstrated that the absorption curve was approximately mono-exponential with an average half time of about 2 hours Joiner (1959 a and b) found that following subcutaneous injections of 0 2 ml (8 i u) of crystalline insulin dissolved in isotonic saline, the absorption occurred more rapidly after injection into the brachial region (half time 68 minutes) than into the femoral region (half time 159 minutes) Similar results were obtained by Nora, Smith and Cameron (1964) Balodimos and Williams (1962) varied the injection volume of dissolved ^{131}I-insulin to which were added varying amounts of carrier insulin They determined the absorption by measuring the amount of radioactivity only at the time of injection and four hours later, and found the residual amount to be 23 per cent of the initial one The injections were given into the femoral and brachial regions but in their results the authors made no distinction between the two sites of injection Table 3 I contains the most important results from the said studies

Joiner (1959 a) observed that the absorption had a tendency to be slow in patients with long duration diabetes – a correlation that Moore and co-workers could not demonstrate. Balodimos and Williams noted that the insulin absorption tended to occur rapidly in patients with high blood sugar concentrations

Binder, Nielsen, and Jorgensen (1967) examined the absorption following subcutaneous injections of two insulin solutions an acid insulin solution, Insulin Novo (SI), and a neutral one, Actrapid (A) The compositions of the

are the capillary membrane permeability for the substance in question, the perfusion rate of blood through the absorbing capillaries, and the affinity of the substance to the subcutaneous tissue The latter factor was examined by adding ^{125}I-insulin (Actrapid) to test tubes containing homogenized subcutaneous tissue and serum The tubes were vigorously shaken in water bath at 37°C for an hour, whereafter the radioactivity of either phase was determined Radioactivity was demonstrable at all examinations in the serum phase only It must consequently be assumed that injected, dissolved insulin spreads in the extracellular volume

Rasio, Hampers, Soeldner, and Cahill (1967) performed simultaneous intravenous injections of glucose, inulin, and insulin in nephrectomized patients with a thoracic duct fistula They found the glucose concentration in the lymph in the thoracic duct to increase significantly during the first minutes after the injection The concentration reached its maximum 7 to 15 minutes after the injection The concentrations of inulin and immunoreactive insulin in the lymph did not increase until 3 to 7 minutes after the injection The maxima for concentrations of inulin and insulin occurred simultaneously 15 to 20 minutes after the injection Following intravenous injections of inulin, dissolved crystalline insuline, and Evans blue, a simultaneous increase was likewise seen in the concentrations of the former two substances in lymph from the thoracic duct The concentration of Evans blue did not rise until 20 minutes after the injection, and it did not reach its maximum during the examination period of 120 minutes The results give reason to suppose that during their passage through the capillary wall, endogenous and exogenous insulins are considerably smaller than albumin and probably also than inulin By examining mesothelium of rats in vitro, Rasio (personal communication) demonstrated that dissolved insulin passes the mesothelium membrane somewhat slower than ^{14}C-inulin and essentially more rapidly than ^{131}I-albumin

The capillary membrane permeability for dissolved insulin thus seems to be of the same order of magnitude as for inulin As inulin likewise is evenly distributed in the extracellular volume, it must be assumed that, at the absorption, insulin has a molecular size not much different from that of inulin (molecular weight 5600) That means that during its passage from tissue to blood, dissolved insulin is probably in a monomeric or dimeric state

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Authors	Insulin	Region	Number of observations	T-50 per cent (min.)	
				mean	S.E.M.
Root et al (1944)	insulin + ^{131}I azobenzene	deltoid	7	appr 480	
Moore et al (1959)	dissolved ^{131}I insulin	brachial	6	142	19
Joiner (1959a)	dissolved ^{131}I insulin	femoral brachial	6 6	159 68	10 5
Nora et al (1964)	dissolved ^{131}I insulin	femoral brachial	? ?	118 62	- -
Binder et al (1967)	Actrapid	femoral brachial	33 17	164 141	7 11
Balodimos and Williams (1962)	dissolved ^{131}I insulin	brachial or femoral	22	23 per cent at site of injection after 4 hours	

Table 3 I Results from previous investigations of the absorption of dissolved insulin

preparations are given in table 1 I The investigation was carried out in diabetic patients The criterions for the diagnosis of diabetes mellitus were in all cases an increased fasting blood sugar and glucosuria Except in a few cases, the diabetes required insulin therapy Table 3 III gives range, mean, and standard error of the mean for age and duration in the various groups of patients examined The ratio between men and women is also stated The injection volume was always 0 3 ml (12 i u) of insulin, and during the period of examination, no other insulin was given In order to avoid reflux into the needle track, the needle rested in the tissue for 15 sec after the injection Injections were not made into lipodystrophic regions Previous injections were disregarded The radioactivity was measured at the site of injection immediately after the injection and every hour for 8 hours

Figure 3 1 shows, on a semilogarithmic scale, the absorption curve determined after injection of Actrapid into the femoral region The curve is characteristic of the absorption of both the acid and the neutral insulin solution, whether injected subcutaneously or intramuscularly

In the following, the absorption course will be described either by this curve or by T-50 per cent, which is defined as the duration of absorption for the first 50 per cent of the injected amount of insulin The value is different from the half time, $t_{1/2}$, for the mono-exponential part of the ab-

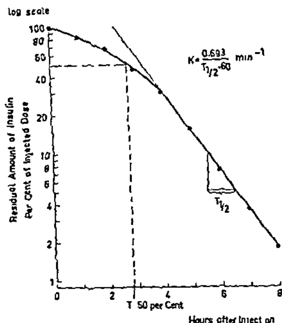


Figure 3 1 Absorption curve following a subcutaneous injection of 0 3 ml of Actrapid into the femoral region.

sorption curve, the latter value is employed for calculation of the rate constant, k, for this part of the curve After the initial phase, the absorption curve – drawn as in figure 3 1 – often had a two-phase course The terminal phase was subtracted prior to calculation of k, therefore, k always represents the mono-exponential course that follows the initial part of the absorption where the absorption rate is increasing

The variation in the absorption course was followed for both SI and A injected into the femoral region An analysis of variance was made on the values for T-50 per cent The results are presented in table 3 II

	SI			A		
Source of variation	df	s^2	F-ratio	df	s^2	F-ratio
Between patients	9	12989	11 2 $p<0\ 001$	4	1930	8 25 $p<0\ 025$
Within patients	10	1159		5	234	
T 50 per cent	251 min.			151 min.		

Table 3 II Variation in T 50 per cent based on duplicate examinations of the absorption in 10 (SI) and in 5 (A) patients.

Authors	Insulin	Region	Number of observations	T-50 per cent (min.)	
				mean	S.E.M
Root et al (1944)	insulin-4-^{131}I azobenzene	deltoid	7	appr 480	-
Moore et al (1959)	dissolved ^{131}I insulin	brachial	6	142	19
Joiner (1959a)	dissolved ^{131}I insulin	femoral brachial	6 6	159 68	10 5
Nora et al (1964)	dissolved ^{131}I insulin	femoral brachial	? ?	118 62	- -
Binder et al (1967)	Actrapid	femoral brachial	33 17	164 141	7 11
Balodimos and Williams (1962)	dissolved ^{131}I insulin	brachial or femoral	22	23 per cent at site of injection after 4 hours	

Table 3 I Results from previous investigations of the absorption of dissolved insulin

preparations are given in table 1 I The investigation was carried out in diabetic patients The criterions for the diagnosis of diabetes mellitus were in all cases an increased fasting blood sugar and glucosuria Except in a few cases, the diabetes required insulin therapy Table 3 III gives range, mean, and standard error of the mean for age and duration in the various groups of patients examined The ratio between men and women is also stated The injection volume was always 0 3 ml (12 i u) of insulin, and during the period of examination, no other insulin was given In order to avoid reflux into the needle track, the needle rested in the tissue for 15 sec after the injection Injections were not made into lipodystrophic regions Previous injections were disregarded The radioactivity was measured at the site of injection immediately after the injection and every hour for 8 hours

Figure 3 1 shows, on a semilogarithmic scale, the absorption curve determined after injection of Actrapid into the femoral region The curve is characteristic of the absorption of both the acid and the neutral insulin solution, whether injected subcutaneously or intramuscularly

In the following, the absorption course will be described either by this curve or by T-50 per cent, which is defined as the duration of absorption for the first 50 per cent of the injected amount of insulin The value is different from the half time, $t_{1/2}$, for the mono-exponential part of the ab-

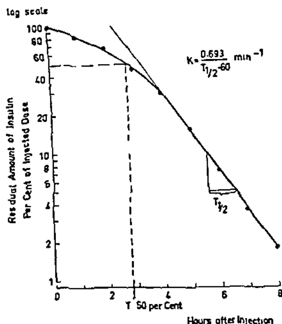


Figure 3 I Absorption curve following a subcutaneous injection of 0 3 ml of Actrapid into the femoral region.

sorption curve, the latter value is employed for calculation of the rate constant, k, for this part of the curve After the initial phase, the absorption curve – drawn as in figure 3 1 – often had a two-phase course. The terminal phase was subtracted prior to calculation of k, therefore, k always represents the mono-exponential course that follows the initial part of the absorption where the absorption rate is increasing

The variation in the absorption course was followed for both SI and A injected into the femoral region An analysis of variance was made on the values for T-50 per cent The results are presented in table 3 II

	SI			A		
Source of variation	df	s^2	F-ratio	df	s^2	F-ratio
Between patients	9	12989	11 2	4	1930	8 25
Within patients	10	1159	$p<0.001$	5	234	$p<0.025$
T 50 per cent	251 min.			151 min		

Table 3.II Variation in T-50 per cent based *on duplicate examinations* of the absorption in 10 (SI) and in 5 (A) patients

Authors	Insulin	Region	Number of observations	T-50 per cent (min.)	
				mean	S.E.M.
Root et al (1944)	insulin 4-^{131}I azobenzene	deltoid	7	appr 480	-
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Joiner (1959a)	dissolved ^{131}I insulin	femoral brachial	6 6	159 68	10 5
Nora et al (1964)	dissolved ^{131}I insulin	femoral brachial	? ?	118 62	- -
Binder et al (1967)	Actrapid	femoral *brachial*	33 *17*	164 *141*	7 *11*
Balodimos and Williams (1962)	dissolved ^{131}I insulin	brachial or femoral	22	23 per cent at site of injection after 4 hours	

Table 3 I Results from previous investigations of the absorption of dissolved insulin

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Figure 3 I shows, on a semilogarithmic scale, the absorption curve determined after injection of Actrapid into the femoral region The curve is characteristic of the absorption of both the acid and the neutral insulin solution, whether injected subcutaneously or intramuscularly

In the following, the absorption course will be described either by this curve or by T-50 per cent, which is defined as the duration of absorption for the first 50 per cent of the injected amount of insulin The value is different from the half time, $t_{1/2}$, for the mono-exponential part of the ab-

For both preparations, the variation within patients was significantly smaller than between patients The reproducibility of T-50 per cent was significantly smaller for SI than for A, the respective coefficients of variation being 13 and 10 per cent

The absorption was examined in 5 regions the femoral, gluteal, abdominal brachial, and scapular region Within the same region, the hourly determinations of the average residual amount always gave smaller values after injection of A than after injection of SI The absorptions of SI and A following injections into the femoral region were investigated in 10 patients At any given time, the residual amount of insulin in the individual patient was always smaller after injection of A than after injection of SI

The results showed great dispersion Four hours after injection of SI into the femoral region, the residual amount of insulin varied from 6 to 67 per cent Similar variations were found for all the regions examined

The regional differences were considerable Thus, after injection of SI into the femoral region, 45 per cent remained 4 hours later, in the gluteal and brachial regions the respective values were 38 and 32 per cent The corresponding values following injections of A were 30, 26, and 23 per cent Four hours after injection of SI into the abdominal and scapular regions, there remained only 16 and 17 per cent, and only 6 and 8 per cent after injection of A

The mean values for T 50 per cent are listed in table 3 III together with the connected 95 per cent confidence limits The table also contains the means of the calculated k values Figure 3 2 gives the values from the table in a blockdiagram and also the 95 per cent confidence limits for the k values

In each region, T-50 per cent for SI was found to exceed that for A. A similar difference could not be demonstrated between the corresponding k values

The femoral, gluteal and brachial regions differed markedly from the abdominal and scapular ones with regard to T-50 per cent as well as to k-value The mutual differences of the former three and the latter two regions were however small

The course of absorption following injections of SI into the femoral and scapular regions in normals did not differ from that seen in diabetics and showed the same regional difference

Figure 3 3 shows the mean absorption rates determined every hour after injections of SI and A into the femoral and abdominal regions Confidence limits 95 per cent During the first 2 hours after injection into the abdominal

Region	Insulin	No of patients	Age (years)	Duration (months)	Ratio men women	T-50% Mean (min)	95% Confidence limits (min)	k value for mono-exponential phase $(min^{-1} \times 10^{-2})$		
								No of patients	Mean	Coefficient of variation (%)
Femoral	SI	37	11–68[1] 34[2] 2.8[3]	0–408[1] 129[2] 19[3]	19 18	223	18	35	0.71	36
	A	33	11–66 29 2.6	0–256 108 16	16 17	164	15	29	0.86	29
Gluteal	SI	16	14–66 38 4.0	1–288 126 24	8 8	204	27	16	0.84	36
	A	17	13–72 44 4.4	9–372 140 21	9 8	155	28	16	1.06	43
Brachial	SI	18	14–51 29 2.7	0–228 91 19	10 8	175	20	18	1.00	46
	A	17	15–73 37 4.8	18–301 139 21	9 8	141	23	17	0.96	27
Abdominal	SI	18	14–66 40 4.0	72–410 178 24	8 10	138	21	18	1.53	39
	A	17	13–69 44 4.0	0–360 116 24	8 9	87	12	16	1.77	27
Scapular	SI	19	12–69 37 4.0	2–288 142 19	8 11	128	21	18	1.62	38
	A	16	21–71 42 3.7	5–319 155 24	8 8	89	14	16	1.64	28

1) Range 2) Mean 3) Standard error of the mean

Table 3 III Data for the patients examined, mean duration of absorption for the first 50 per cent of the injected insulin (T-50 *per cent*), and the average values of k for the mono-exponential part of the absorption curve. The absorption rate varied in some cases so much that calculation of the

injected insulin was used as a parameter for the absorption, the other parameters characterizing the patients were age, sex, diet, duration of diabetes, heredity, retinopathy, nephropathy, sense of vibration, the activity of the patient during the examination (whether confined to bed or not), injection region, skinfold thickness, insulin preparation, and fasting blood sugar on the day of examination The material consisted of 73 patients The sense of vibration was determined on the plantar side of the big toe with a biothesiometer (Bio-Medical Instrument Company, Chagrin Falls, Ohio). The preparations employed were Insulin Novo and Actrapid, the sites of injection were the femoral and scapular regions

The following multiple regression equation expresses the relationship between T-50 per cent and the variables found significant from the stepwise analysis

$$Y = 4\,79 - 1\,72x_r - 0\,99x_I - 0\,080x_v + 0\,00081x_a \cdot x_v \qquad (1)$$

where Y is the best fitted value for T-50 per cent according to the method of least squares, x_r is zero for injection into the femoral region and one for injection into the scapular region, x_I is equal to zero for SI and to one for A, x_v is the sense of vibration expressed in volts, and x_a is the patient's age in years It appears from the equation that a decreasing sense of vibration

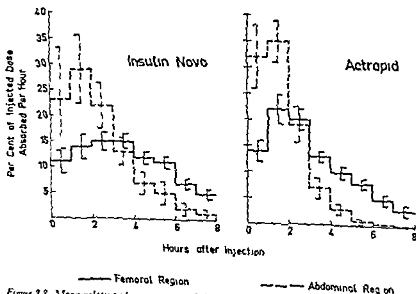


Figure 3 9 Mean relative absorption rates following subcutaneous injections of Insulin Novo and Actrapid into two different regions. Confidence limits 95 per cent

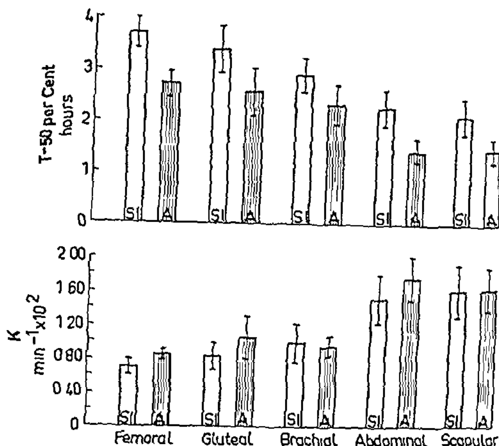


Figure 3 2 Blockdiagrams of the values given in table 3 III for T 50 per cent and for k the rate constant for the mono exponential part of the absorption curve Confidence limits 95 per cent

region, the absorption rate was approximately twice that found in the femoral region

The course of absorption was independent of the patients being confined to bed or not during the examinations

At 58 determinations of the absorption from the femoral region, simultaneous measurements were made of skin temperature at the site of injection, room temperature, and degree of humidity The skin temperature varied from 24 4 to 27 8°C, the room temperature from 21 to 25°C, and the relative humidity from 66 to 77 per cent The absorption course was not correlated to any of those parameters

By employing a stepwise, multiple regression technique as described by Efroymson (1960), Nielsen and Binder (1967) examined in diabetics whether the absorption of dissolved insulin injected subcutaneously was correlated to certain parameters The duration of absorption for 50 per cent of the

Muscle	Insulin	No. of patients	T-50% Mean (min.)	95% Confidence limits (min.)	k-value for mono-exponential phase ($min^{-1} \times 10^{-2}$)		
					No. of patients	Mean	Coefficient of variation (%)
anterior tibial	SI	9	173	31	9	0.86	38
straight femoral	SI	8	132	33	7	1.22	33
	A	8	89	31	8	1.64	31
middle gluteal	SI	11	110	22	11	1.61	42
deltoid	SI	8	86	31	8	1.95	22
	A	8	69	12	8	1.96	12

Table 3 IV Mean duration of absorption for the first 50 per cent of the injected insulin (T 50 per cent) and the average values of k for the mono-exponential part of the absorption curve The absorption rate varied in a single case so much that calculation of the k value could not be made in the ordinary way

ing intramuscular injection was also found occasionally to have a slow terminal phase The k-values given in the table and figure correspond to the initial mono-exponential phase of the curve In table 3 IV are listed the mean values for T-50 per cent and the calculated values of k. Figure 3 5 gives these values with 95 per cent confidence limits The values for the absorption after subcutaneous injection into the same regions are sketched in on the figure Figure 3 4 shows the mean values for absorption after subcutaneous and intramuscular injections of SI into the femoral region

As confinement to bed was found not to influence the absorption, no distinction with regard to the patients' activity was made when presenting the results from the femoral and the deltoid muscles

Discussion

The investigations showed that at a given time, the residual amount of insulin at the site of injection of SI always exceeded that found after injection of A For most regions the same difference was apparent in the values for T-50 per cent, but not in those for k It follows that the difference must be connected with the initial part of the absorption

According to Bermont (1967), intradermal injection of insulin in acid solution induces a visible flush, a reaction not seen after injection of insulin in neutral solution Hereupon it can be assumed that application of the acid solution causes a further initial depression of the absorption of SI

During neutralization in the tissue of the acid insulin solution, a precipi-

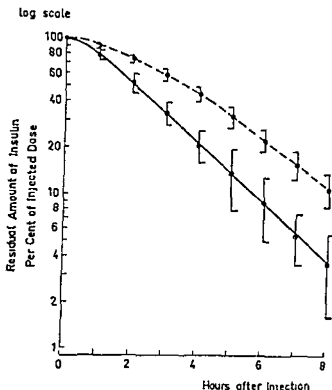


Figure 3 4 Mean values for absorption after subcutaneous (dashed curve) and intramuscular (solid curve) injections of 0 3 ml of SI into the femoral region Confidence limits 95 per cent

(x_V increasing) corresponds to a faster absorption (Y diminishing) if the other variables are kept constant The last term of the equation shows that this correlation, however, is age dependent, being most pronounced in young patients and diminishing with increasing years

Intramuscular injections

The absorption following intramuscular injections was examined with the same two preparations as that following subcutaneous injections The injection of 0 3 ml (12 i u) was given at the same site as was the subcutaneous one

The absorption was examined in four muscles the middle gluteal, the anterior tibial, the straight femoral, and the deltoid muscle In the case of the first two muscles, the patients were confined to bed during the whole examination For determining the absorption from the other two muscles, half the patients were confined to bed – lying as quietly as possible The physical activity of the other half was moderate, though not defined

The absorption following intramuscular injections had a course similar to that after subcutaneous ones the relative absorption rate rose initially to become constant after some time (figure 3 4) The absorption curve follow-

Muscle	Insulin	No of patients	T-50% Mean (min.)	95% Confidence limits (min.)	k-value for mono-exponential phase (min⁻¹ × 10⁻²)		
					No. of patients	Mean	Coefficient of variation (%)
anterior tibial	SI	9	173	31	9	0 86	38
straight femoral	SI	8	132	33	7	1 22	33
	A	8	89	31	8	1 64	31
middle gluteal	SI	11	110	22	11	1 61	42
deltoid	SI	8	86	31	8	1 95	22
	A	8	69	12	8	1 96	12

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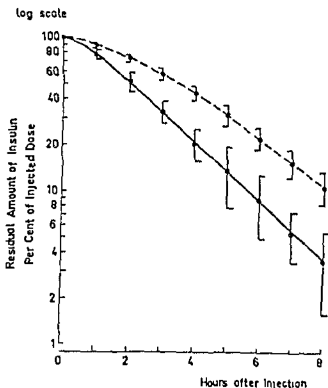


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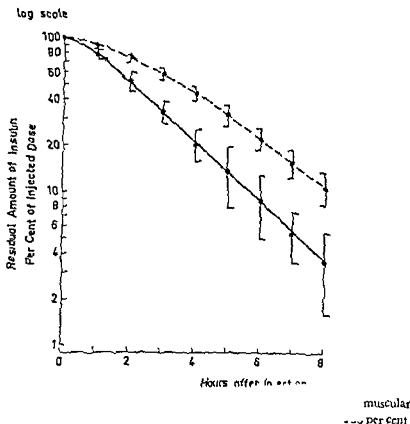
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muscular

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The absorption following intramuscular injections had a course similar to that after subcutaneous ones the relative absorption rate rose initially to become constant after some time (figure 3 4) The absorption curve follow-

tions of SI and A probably is due to a local effect on the capillaries triggered off by the acid injection fluid

The regional differences demonstrated in the absorption of dissolved insulin, made Munck, Andersen, and Binder (1967) examine the absorption of ^{125}I antipyrine following subcutaneous injection into various regions The capillary membrane permeability for antipyrine has such a size that its clearance is limited by the blood flow only (Renkin 1955) Lindbjerg (1967) found the same ratio of ^{133}Xe clearance to ^{125}I-antipyrine clearance in the resting muscle as in the maximally hyperemic muscle This suggests that iodine-labelled antipyrine is suitable for blood flow measurements

Munck et al calculated the rate constant for the initial, mono-exponential part of the absorption curve after injection of ^{125}I-antipyrine The respective averages for the femoral, scapular, and abdominal regions were in normals 0 066 min $^{-1}$, 0 140 min $^{-1}$, and 0 102 min^{-1} There was a significant ($p <$ 0 05) difference between the rate constant for the femoral region and those for the scapular and abdominal regions There was no significant mutual difference between the two latter ones The ratios between the rate constants thigh back and thigh abdomen for ^{125}I-antipyrine and for the two insulin preparations are given with 95 per cent confidence limits in table 3 V

	^{125}I AP	SI	A
$\frac{k_{femoral}}{k_{scapular}}$	0 47 (0 17)	0 44 (0 11)	0.52 (0 11)
$\frac{k_{femoral}}{k_{abdominal}}$	0 65 (0 19)	0 46 (0 11)	0 49 (0 10)

Table 3 V The ratios between rate constants for ^{125}I-antipyrine and for preparations after injections into various subcutaneous regions The value for ^{125}I antipyrine are from Munck, Andersen and Binder (1967)

As for ^{125}I-antipyrine, the rate constant is dependent on the blood flow in the capillaries and the capillary surface of the absorbing tissues, in the case of insulins, however, the rate constant is also conditioned by possible differences in the capillary membrane permeability for insulin

The calculated ratios did not show any significant differences This means that with regard to insulin, there is no difference between the capillary membrane permeabilities in the regions concerned It also means that the regional differences in the absorption of dissolved insulin are conditioned by regional differences in perfusion

In the present investigation, the insulin absorption differed but little

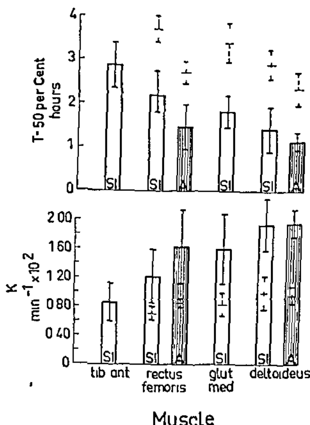


Figure 3 5 Blockdiagrams of the values given in table 3 IV for T 50 per cent and for the rate constant for the mono exponential part of the absorption curve Confidence limits 95 per cent The corresponding values from subcutis are inserted

tation of insulin will take place at intermediate pH values (Schlichtkrull 1958) As mentioned by Binder, Nielsen, and Jørgensen (1967), this precipitation might have a delaying effect on the absorption of SI It is, however, probable that the buffer capacity of the tissue is so great that the change over of pH occurs very fast When no other factors inhibit the re-dissolution of insulin, it will take place at the same rate as the change-over of pH The presence of Zn^{++} may present such an inhibition However, the absorption following injection of SI 'without zinc' did not differ from that found after injection of SI containing zinc (see page 34) Schlichtkrull observed that the hypoglycemic effect of crystalline insulin dissolved at pH=3 was independent of the Zn^{++} content Thus, also this finding makes it unlikely that the Zn^{++} content in the insulin solution is of importance for the initial depression of the absorption being more pronounced for SI than for A

It must therefore be concluded that the difference between the absorp

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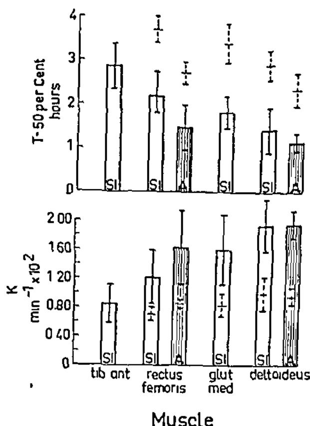


Figure 3.5 Blockdiagrams of the values given in table 3.IV for T-50 per cent and for the rate constant for the mono-exponential part of the absorption curve. Confidence limits 95 per cent. The corresponding values from subcutis are inserted

tation of insulin will take place at intermediate pH values (Schlichtkrull 1958). As mentioned by Binder, Nielsen, and Jorgensen (1967), this precipitation might have a delaying effect on the absorption of SI. It is, however, probable that the buffer capacity of the tissue is so great that the change-over of pH occurs very fast. When no other factors inhibit the re-dissolution of insulin, it will take place at the same rate as the change-over of pH. The presence of Zn^{++} may present such an inhibition. However, the absorption following injection of SI 'without zinc' did not differ from that found after injection of SI containing zinc (see page 34). Schlichtkrull observed that the hypoglycemic effect of crystalline insulin dissolved at pH=3 was independent of the Zn^{++} content. Thus, also this finding makes it unlikely that the Zn^{++} content in the insulin solution is of importance for the initial depression of the absorption being more pronounced for SI than for A.

It must therefore be concluded that the difference between the absorp-

correlation between the temperatures measured and the course of absorption The room temperature varied between 21 and 25°C

The multiple regression analysis showed that dissolved insulin was absorbed more rapidly with decreasing sense of vibration (cf page 43), whereas the degree of retinopathy and the frequency of nephropathy were not significantly correlated to T-50 per cent

A greater absorption rate may be due to an increased capillary membrane permeability for insulin Trap-Jensen, Alpert, Rio, and Lassen (1967) showed that the diffusion capacity of the capillaries for Na^+ was larger in patients with diabetes of long duration than in patients with newly discovered diabetes. The latter ones had the same diffusion capacity as normals As it must be assumed that insulin passes through the capillary wall by the same route as does Na^+, T-50 per cent would be expected to fall with increasing duration of diabetes However, in the present material, the mean duration of diabetes was shorter than in that of Trap-Jensen et al , namely approximately 10 years against 24 It therefore displayed a higher frequency of angiopathy, 50 per cent of the patients in the present work had retinopathy and some 20 per cent nephropathy against 100 per cent and 50 per cent in the above mentioned material Thus, the results might indicate that the sense of vibration is better correlated to the degree of microangiopathy than is the duration of diabetes

B ukr 4

between the muscles examined, though the absorption from the anterior tibial muscle was slower than that from the other three muscles

The results showed that the absorption was faster following injections of dissolved insulin into the various muscles than when the injections were given into the corresponding subcutaneous tissues This finding does not agree with those of Moore, Mitchell and Chalmers (1959) and of Nora Smith and Cameron (1964) These investigators could not demonstrate any difference between subcutaneous and intramuscular absorption within the same region There is no explanation of this discrepancy

The average absorption course from the middle gluteal muscle which is the one preferred in therapy, was not significantly different from the average courses found after subcutaneous injections into the back or the abdomen (figure 3 2) The variation in absorption between patients was the same after intramuscular as after subcutaneous injections

It was surprising that the absorption – especially after intramuscular injections – did not differ demonstrably between the group confined to bed and that with moderate physical activity This indicates that the capillary density is not essentially augmented during moderate muscular exercise

Reported data on absorption of subcutaneously injected insulin are listed in table 3 I The preparation employed by Root, Irvine Evans Reiner, and Carpenter (1944) must be considered too modified for their results to be taken as representative of the absorption of dissolved crystalline insulin Balodimos and Williams (1962) as well as Nora et al (1964) have not provided enough information about the properties of the ^{131}I-insulin solutions employed Therefore, it is not relevant to compare the present results with theirs

Moore et al (1959) and Joiner (1959 a and b) used ^{131}I-insulin in neutral solution the results from the subcutaneous injections of Actrapid can therefore be compared with theirs As for injections of Actrapid into the femoral region the results are in good agreement with those found in the same region by Joiner With regard to the brachial region Binder et al (1967) found the average value of T 50 per cent to be 141 min which value is consistent with that reported by Moore et al but not with Joiner s result who in this region found T 50 per cent to average only 68 min Joiner examined all his patients at a room temperature of 32° C It is probable that this has caused more capillaries to be open in the brachial region than in the femoral one

At many examinations of the absorption from the femoral region the room temperature and the skin temperature were measured simultaneously with each determination of the residual amount of insulin There was no

correlation between the temperatures measured and the course of absorption. The room temperature varied between 21 and 25°C.

The multiple regression analysis showed that dissolved insulin was absorbed more rapidly with decreasing sense of vibration (cf. page 43), whereas the degree of retinopathy and the frequency of nephropathy were not significantly correlated to T-50 per cent.

A greater absorption rate may be due to an increased capillary membrane permeability for insulin. Trap-Jensen, Alpert, Rio, and Lassen (1967) showed that the diffusion capacity of the capillaries for Na^+ was larger in patients with diabetes of long duration than in patients with newly discovered diabetes. The latter ones had the same diffusion capacity as normals. As it must be assumed that insulin passes through the capillary wall by the same route as does Na^+, T 50 per cent would be expected to fall with increasing duration of diabetes. However, in the present material, the mean duration of diabetes was shorter than in that of Trap-Jensen et al., namely approximately 10 years against 24. It therefore displayed a higher frequency of angiopathy, 50 per cent of the patients in the present work had retinopathy and some 20 per cent nephropathy against 100 per cent and 50 per cent in the above mentioned material. Thus, the results might indicate that the sense of vibration is better correlated to the degree of microangiopathy than is the duration of diabetes.

Chapter 4

ABSORPTION OF INSULIN PREPARATIONS WITH PROTRACTED ACTION

Although Abel in 1926 succeeded in isolating insulin in its crystalline form, only Scott's (1934) observation of the importance of zinc ions for the crystallization process rendered it possible to produce a well defined crystalline insulin preparation During the years 1934 to 1940 many studies were carried out to investigate the influence of added zinc ions on the therapeutic effect of insulin solutions Scott and Fisher (1935) demonstrated that adding large quantities of zinc ions (0 4 to 1 0 mg of Zn^{++} per unit of insulin) produced a protracted effect Similar results were obtained by Bischoff and Jemtegaard (1937) and by Blatherwick, Ewing, and Bradshaw (1938) Due to the high proportion of zinc ions, these preparations could not be used in therapy, and efforts were focused on other methods of protracting the effect of insulin preparations Aubertin, Servantie, and Chassagnette (1938) demonstrated that a suspension of amorphous insulin containing no more than 2 to 14 μg of Zn^{++} per unit of insulin had a prolonging effect, but this finding had no therapeutic consequences

The above studies on the influence of the zinc ion resulted in the said method of insulin crystallization and consequently in a more well defined insulin standard Another result was the application of zinc ions as a stabilizer in preparations where the prolonged effect had been otherwise produced

When in 1936 Hagedorn, Normann Jensen, Krarup, and Wodstrup succeeded in preparing an insulin with a protracted action, the protamine insulin, it marked a turning-point in the treatment of diabetes mellitus Instead of multiple daily injections of insulin in acid solution, two – in mild cases one – injections a day were now sufficient (Krarup 1935)

The same year, Scott and Fisher (1936) showed that adding Zn^{++} to protamine insulin produced a further protracted effect

Protamine insulin and protamine zinc insulin soon became the insulin preparations most used Their administration had, however, certain drawbacks Thus, protamine insulin was unstable in neutral suspensions so that the patients themselves had to mix protamine and insulin with a buffer

Though the effect of protamine zinc insulin was long, it was not sufficient, *and a supplementary* dose of ordinary insulin was therefore often necessary

These difficulties were overcome in Hagedorn's laboratory by Krayenbuhl and Rosenberg (1946), who prepared a stable, neutral suspension of protamine insulin NPH® (neutral protamine Hagedorn) or 'isophan insulin'

A detailed survey of the various insulin preparations and their chemical and pharmacological properties was given by Schlichtkrull (in press)

In the early fifties, the Novo laboratories took up anew the study of zinc insulin preparations It was found that, in the absence of protamine, *the phosphate buffer inhibited the zinc ions in precipitating insulin* By omitting the phosphate buffer, stable preparations were obtained in which reduction of the amount of protamine shortened the time of action (Schlichtkrull 1958) The preparations had a protracted effect even when no protamine was added The precipitated, amorphous insulin was used for the preparation Semilente

The degree of protraction was, however, not sufficient for applying the preparation to therapy consisting of one daily injection A considerable degree of protraction was obtained at the same zinc concentration (2 µg of Zn^{++} per unit of insulin) by substituting insulin crystals for the amorphous insulin The preparation was called Ultralente When Semilente and Ultralente were mixed, stable preparations with an intermediate action were obtained Lente, the mixture chosen for therapeutic application, consists of the two preparations in a proportion of 3 7 (Hallas Møller, Jersild, Petersen, and Schlichtkrull 1954)

In 1958, Schlichtkrull described in detail the chemical properties of these preparations He also advanced a method for preparing specific insulin crystals with a lower Zn^{++} content (0 33 µg Zn^{++} per unit of insulin) The crystals had a considerable protracting effect when injected in suspension Containing no precipitants – such as protamine or zinc ions – the suspensions can be mixed with a quick acting insulin without the effect of the latter being changed The method resulted in preparation of Rapitard *where* 75 per cent of the insulin are suspended beef insulin crystals and 25 per cent are dissolved quick-acting pig insulin (Schlichtkrull, Munck, and Jersild 1965)

Absorption of preparations containing one insulin component

This investigation comprised the following preparations Semilente (SL), Ultralente (UL), and C II, the crystalline component in Rapitard The compositions of the insulins are shown in table I I, and the processes of *preparation* are described on page *12*

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The same year, Scott and Fisher (1936) showed that adding Zn^{++} to protamine insulin produced a further protracted effect.

Protamine insulin and protamine zinc insulin soon became the insulin preparations most used. Their administration had, however, certain drawbacks. Thus, protamine insulin was unstable in neutral suspensions so that the patients themselves had to mix protamine and insulin with a buffer

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Absorption of preparations containing one insulin component

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Case No.	Age (years)	Duration (months)	Residual amount of insulin in percentages of injected dose (hours after injection)											k-value for mono exponential part of curve (m n $^{-1}$ × 10 $^{-2}$)
			1	2	3	4	5	6	7	8	15	24	27	
510 – 326	16	156	96	88	80	73	64	58	51	43	8 5	1 2	0 8	0 43
541 – 327	15	37	98	94	84	78	72	61	50	46	8 8	1 2	0 9	0 39
551 – 336	39	106	95	94	88	79	66	57	51	40	4 8	1 1	0 8	0 44
552 – 337	46	370	97	90	84	77	65	58	50	44	9 8	0 7	–*)	0 23
557 – 341	33	276	100	96	94	86	79	76	69	63	28	2 1	1 2	0 53
558 – 342	31	168	100	94	90	82	78	72	64	59	23	1 6	1 2	0 39
Mean	30	186	98	93	87	79	71	64	56	49	14	1 3	1 0	0 40
Coefficient of variation (per cent)			2	3	6	6	9	13	15	19	67	39	20	55
95 per cent confidence limits			2 2	3 1	5 3	4 7	7 0	8 6	8 8	9 9	9 8	0 5	0 2	0 23

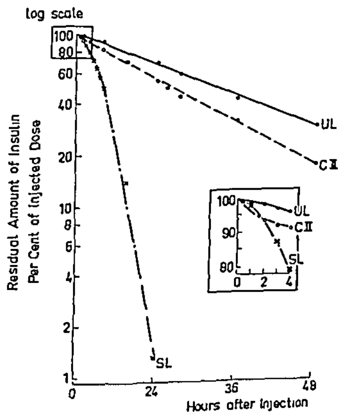
*) No determination

Table 4 1 Absorption of Semilente following subcutaneous injection of one ml (40 i u) into the femoral region

In all cases, one ml of insulin (40 i.u.) was injected subcutaneously into the femoral region All examinations started at 8. The patients had their usual diet and were not confined to bed. None had lipodystrophy or other cutaneous symptoms. Since their diabetes mellitus was discovered, all patients had been treated with insulin Ages of patients and duration of their diabetes are given in tables 4 I–III.

The residual amount of insulin was determined every hour for the first 8 hours after the injection, thereafter – at varying intervals – until up to 111 hours had elapsed

The results are presented in tables 4 I to III. Determination was impossible in some cases. No mean value was calculated, if, at a given time after the injection, more than one value was missing. The average absorption curves for the three preparations are shown in figure 4 1



[illegible] absorption curves following subcutaneous injections of [illegible]

first 4 hours is inserted

Case No.	Age (years)	Duration (months)	Residual amount of insulin in percentages of injected dose (hours after injection)											k value for mono-exponential part of curve (min⁻¹ × 10⁻²)
			1	2	3	4	5	6	7	8	15	24	27	
510 – 326	16	156	96	88	80	73	64	58	51	43	8 5	1 2	0 8	0 43
541 – 327	15	37	98	91	84	78	72	61	50	46	8 8	1 2	0 9	0 39
551 – 336	39	106	95	94	80	79	66	57	51	40	4 8	1 1	0 8	0 44
552 – 337	46	370	97	90	81	77	65	58	50	44	9 8	0 7	–*)	0 23
557 – 341	33	276	100	96	94	86	79	76	69	63	28	2 1	1 2	0 53
558 – 342	31	168	100	94	90	82	78	72	64	59	23	1 6	1 2	0 39
Mean	30	186	98	93	87	79	71	64	56	49	14	1 3	1 0	0 40
Coefficient of variation (per cent)			2	3	6	6	9	13	15	19	67	39	20	55
95 per cent confidence limits			2 2	3 1	5 3	4 7	7 0	8 6	8 8	9 9	9 8	0 5	0 2	0 23

*) No determination

Table 4 1 Absorption of Semilente following subcutaneous injection of one ml (40 i u) into the femoral region

In all cases, one ml of insulin (40 i u) was injected subcutaneously into the femoral region All examinations started at 8 The patients had their usual diet and were not confined to bed None had lipodystrophy or other cutaneous symptoms Since their diabetes mellitus was discovered, all patients had been treated with insulin Ages of patients and duration of their diabetes are given in tables 4 I–III

The residual amount of insulin was determined every hour for the first 8 hours after the injection, thereafter – at varying intervals – until up to 111 hours had elapsed

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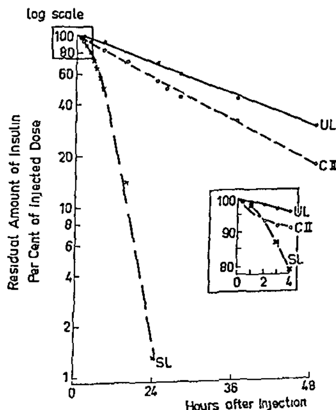


Figure 4 1 The respective average absorption curves following subcutaneous injections of Semilente (SL), the crystalline component in Rapitard (C II), and Ultralente (UL) The curves correspond to the values given in tables 4 I–III An enlargement of the curves for the first 4 hours is inserted

Case No	Age (years)	Duration (months)	Residual amount of insulin in percentages of injected dose (hours after injection)												
			1	2	3	4	8	15	24	32	48	72	96	103	111
536 – 323	38	204	99	95	95	93	88	–*)	81	72	30	11	5	4	4
537 – 324	37	172	102	100	100	99	94	–*)	94	92	84	68	48	39	33
542 – 328	21	54	97	99	98	97	93	93	92	92	86	81	77	77	75
543 – 329	67	96	96	94	92	92	84	79	81	79	72	53	44	42	40
547 – 333	60	456	100	99	98	98	95	88	71	58	35	21	11	10	8
548 – 140	44	48	97	100	94	92	91	50	22	12	3	1	–*)	–*)	–*)
553 – 338	52	72	99	96	97	95	91	83	69	46	25	13	7	6	5
554 – 020	23	102	97	101	100	98	95	85	74	64	51	30	20	18	16
559 – 313	13	12	99	93	99	98	92	71	37	25	13	7	4	–*)	–*)
560 – 314	34	240	98	98	98	98	91	85	73	49	16	3	1	–*)	–*)
Mean	39	146	99	98	97	96	91	–	69	59	42	29	24	–	–
Coefficient of variation (per cent)			2	4	4	3	8	–	33	45	73	100	109	–	–
95 per cent confidence limits			1	3	3	2	5	–	10	16	19	22	21	–	–

*) No determination

Table 4 III Absorption of Ultralente following subcutaneous injection of one ml (40 i u) into the femoral region

Case No.	Age (years)	Duration (months)	Residual amount of insulin in percentages of injected dose (hours after injection)											
			1	2	3	4	8	15	24	27	31	48	72	87
353 – 261	19	81	98	91	91	91	69	51	29	23	22	–*)	–*)	–*)
538 – 106	25	110	95	91	87	86	79	62	48	42	33	15	6 2	3 9
539 – 325	35	150	99	91	89	89	78	53	38	34	27	16	6 5	3 8
519 – 334	61	132	98	96	96	95	86	75	56	51	44	22	8 3	4 9
550 – 335	34	160	97	93	90	90	86	80	71	68	66	56	43 0	36 0
555 – 339	29	43	97	94	90	87	86	78	61	54	50	29	13 8	8 0
556 – 310	63	204	99	98	98	96	92	92	72	70	66	46	28 0	20 0
Mean	38	126	98	93	92	91	82	70	54	49	44	31	17 6	12 8
Coefficient of variation (per cent)			2	3	4	4	9	22	18	35	40	54	85	101
95 per cent confidence limits			1	3	4	4	7	14	9	16	16	16	16	14

*) No determination

Table 4 11 Absorption of the crystalline component in Rapitard (C II) following subcutaneous injection of one ml (40 ı u) into the femoral region

Case No	Age (years)	Duration (months)	Residual amount of insulin in percentages of injected dose (hours after injection)												
			1	2	3	4	8	15	24	31	48	72	96	103	111
536 – 323	38	204	99	95	95	93	88	–*)	81	72	30	11	5	4	4
537 – 324	37	172	102	100	100	99	94	–*)	91	92	84	68	48	39	33
542 – 320	21	54	97	99	98	97	93	93	92	92	86	81	77	77	75
543 – 329	67	96	96	94	92	92	84	79	81	79	72	53	44	42	40
547 – 333	60	456	100	99	98	98	95	88	71	58	35	21	11	10	8
548 – 140	44	48	97	100	94	92	91	50	22	12	3	1	–*)	–*)	–*)
553 – 338	52	72	99	96	97	95	91	83	69	46	25	13	7	6	5
554 – 020	23	102	97	101	100	98	95	85	74	64	51	30	20	18	16
559 – 343	13	12	99	99	99	98	92	71	37	25	13	7	4	–*)	–*)
560 – 344	31	240	99	98	98	98	91	85	73	49	16	3	1	–*)	–*)
Mean	39	146	99	98	97	96	91	–	69	59	42	29	24	–	–
Coefficient of variation (per cent)			2	4	4	3	8	–	33	45	73	100	109	–	–
95 per cent confidence limits			1	3	3	2	5	–	10	16	19	22	21	–	–

*) No determination

Table 4 III Absorption of Ultralente following subcutaneous injection of one ml (40 i u) into the femoral region

Case No.	Age (years)	Duration (months)	Residual amount of insulin in percentages of injected dose (hours after injection)											
			1	2	3	4	8	15	24	27	31	48	72	87
353 – 261	19	81	98	91	91	91	69	51	29	23	22	–*)	–*)	–*)
538 – 106	25	110	95	*91*	*87*	*86*	*79*	*62*	*48*	*42*	*33*	*15*	*6.2*	*3.9*
539 – 325	35	150	99	91	89	89	78	53	38	34	27	16	6.5	3.8
519 – 334	61	132	98	96	96	95	86	75	56	51	44	22	8.3	4.9
550 – 335	34	160	97	93	90	90	86	80	71	68	66	56	43.0	36.0
555 – 339	29	43	97	94	90	87	86	78	61	54	50	29	13.8	8.0
556 – 340	63	204	99	98	98	96	92	92	72	70	66	46	28.0	20.0
Mean	38	126	98	93	92	91	82	70	54	49	44	31	17.6	12.8
Coefficient of variation (per cent)			2	3	4	4	9	22	18	35	40	54	85	101
95 per cent confidence limits			1	3	4	4	7	14	9	16	16	16	16	14

*) No determination

Table 4 II Absorption of the crystalline component in Rapitard (C II) following subcutaneous injection of one ml (40 i. u.) into the femoral region

Absorption of preparations containing two insulin components

The investigations of the absorptions of Ultralente and C II showed that they lasted far more than 24 hours The two components form the greater part of Lente and Rapitard, respectively So it was only to be expected that it would be neccessary to administer these preparations for several successesive days in order to obtain 24 hours where the total amount of absorbed insulin could be accounted for with a reasonable accuracy

The absorptions of Lente and Rapitard were investigated in 12 patients (ages 16 to 56 years, duration of diabetes 5 to 34 years) All patients had been treated with insulin since their diabetes was discovered, and in all the cases, the diabetes was difficult to regulate None had lipodystrophy or other cutaneous symptoms

The absorption was examined after injections into the femoral and abdominal regions Two of the four possible combinations of insulin and region were examined in each patient Each examination of the absorption of the labelled insulin lasted for 96 hours, preceded by at least 72 hours where the corresponding unlabelled insulin was given into the region in question The sequence of these 7 injections is shown in figure 4 2 In order to obtain a stable adjustment of the patients with regard to insulin and diet, no examination was made until at least 4 days after admission to hospital The insulin was given by a single daily injection at 8 Each patient s insulin dose did not vary more than ± 4 i u Between patients, the dose varied from 30 to 44 i u a day, corresponding to from 0 75 to 1 1 ml

The patients were given a standard diet of 2,000 calories consisting of 200 g carbohydrates, 100 g fat, and 90 g protein Breakfast was given at 8, lunch at noon, tea at 15, and dinner at 18 The blood sugar concentration was determined (Hagedorn and Normann Jensen 1923) at 8, 9, 12, 15, 18, 21, and 2

The residual amounts of insulin were determined at all sites of injection at 8, 11, 15, 19, 21, 23, 2, and 5, during the 4-day examination period, and furthermore at 8, 11, and 15 on the fifth day During the first day after each injection, additional determinations were made at the site in question at 9, 10, 12, 13, 14, and 18 Thus, the absorption of the first injection was followed for 103 hours, and those of the subsequent three injections for 79, 55, and 31 hours, respectively

Figures 4 3 and 4 4 show the individual absorption curves after injections into the femoral region of Lente and Rapitard, respectively In all cases there elapsed more than 48 hours before an individual dose of Lente was absorbed The absorption of the individual doses was often irregular from

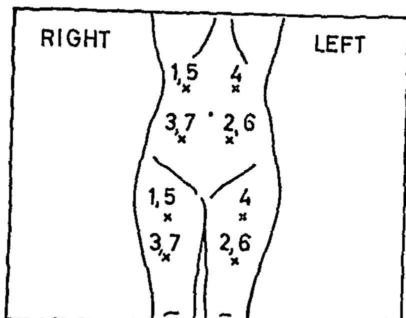


Figure 4 2 Sequence of injections of Lente and Rapitard described in the text An examination period lasted for 7 days where all injections were given into the same region, during the first 3 days, unlabelled insulin was injected in the sequence 1-3, during the last 4 days, the corresponding ^{131}I-insulin was given in the sequence 4-7

The absorption curve following injection of Semilente differed clearly from those of the two other preparations The shape of the curve with its initial rise was similar to that found after injection of dissolved insulin (see figure 3 1) The relative absorption rate became constant after approximately 8 hours The first 50 per cent of the injected amount of SL were absorbed on an average after 8 hours The k-value for the mono-exponential part of the curve averaged 0 40 min^{-1} 10^{-2}

During the first hours, the average absorption curves for UL and C II were approximately mono-exponential with half times about 30 and 40 hours The two preparations differed in their initial courses in that UL had a somewhat slow initial absorption rate, whereas that of C II was clearly higher Beyond the first hour after the injection, no significant difference was demonstrable between UL and C II

It was characteristic of the individual absorption courses after injection of UL that, for many hours often, no fall could be measured in the residual amount of insulin Though to a smaller degree, the same phenomenon also occurred after injection of C II

The variation between the individual absorption curves was essentially smaller after injection of SL than after injection of UL or C II

Absorption of preparations containing two insulin components

The investigations of the absorptions of Ultralente and C II showed that they lasted far more than 24 hours. The two components form the greater part of Lente and Rapitard, respectively So it was only to be expected that it would be neccessary to administer these preparations for several successesive days in order to obtain 24 hours where the total amount of absorbed insulin could be accounted for with a reasonable accuracy

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The patients were given a standard diet of 2,000 calories consisting of 200 g carbohydrates, 100 g fat, and 90 g protein Breakfast was given at 8, lunch at noon tea at 15 and dinner at 18 The blood sugar concentration was determined (Hagedorn and Normann Jensen 1923) at 8, 9, 12, 15, 18, 21, and 2

The residual amounts of insulin were determined at all sites of injection at 8 11, 15, 19, 21, 23, 2, and 5, during the 4-day examination period, and furthermore at 8, 11, and 15 on the fifth day During the first day after each injection additional determinations were made at the site in question at 9 10, 12 13, 14, and 18 Thus, the absorption of the first injection was followed for 103 hours, and those of the subsequent three injections for 79 55, and 31 hours, respectively

Figures 4 3 and 4 4 show the individual absorption curves after injections into the femoral region of Lente and Rapitard, respectively In all cases there elapsed more than 48 hours before an individual dose of Lente was absorbed The absorption of the individual doses was often irregular from

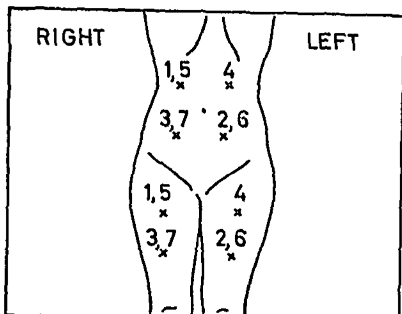


Figure 4 2 Sequence of injections of Lente and Rapitard described in the text An examina tion period lasted for 7 days where all injections were given into the same region during the first 3 days unlabelled insulin was injected in the sequence 1–3 during the last 4 days the corresponding ^{125}I insulin was given in the sequence 4–7

The absorption curve following injection of Semilente differed clearly from those of the two other preparations The shape of the curve with its initial rise was similar to that found after injection of dissolved insulin (see figure 3 1) The relative absorption rate became constant after approximately 8 hours The first 50 per cent of the injected amount of SL were absorbed on an average after 8 hours The k-value for the mono exponential part of the curve averaged 0 40 min $^{-1}$ 10^{-2}

During the first hours, the average absorption curves for UL and C II were approximately mono exponential with half times about 30 and 40 hours The two preparations differed in their initial courses in that UL had a somewhat slow initial absorption rate, whereas that of C II was clearly higher Beyond the first hour after the injection, no significant difference was demonstrable between UL and C II

It was characteristic of the individual absorption courses after injection of UL that, for many hours often, no fall could be measured in the residual amount of insulin Though to a smaller degree, the same phenomenon also occurred after injection of C II

The variation between the individual absorption curves was essentially smaller after injection of SL than after injection of UL or C II

Absorption of preparations containing two insulin components

The investigations of the absorptions of Ultralente and C II showed that they lasted far more than 24 hours The two components form the greater part of Lente and Rapitard, respectively So it was only to be expected that it would be neccessary to administer these preparations for several successesive days in order to obtain 24 hours where the total amount of absorbed insulin could be accounted for with a reasonable accuracy

The absorptions of Lente and Rapitard were investigated in 12 patients (ages 16 to 56 years duration of diabetes 5 to 34 vears) All patients had been treated with insulin since their diabetes was discovered, and in all the cases, the diabetes was difficult to regulate None had lipodystrophy or other cutaneous symptoms

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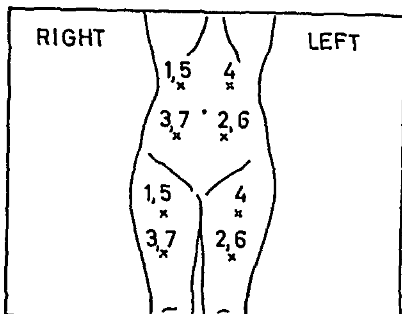


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It was characteristic of the individual absorption courses after injection of UL that, for many hours often, no fall could be measured in the residual amount of insulin Though to a smaller degree, the same phenomenon also occurred after injection of C II

The variation between the individual absorption curves was essentially smaller after injection of SL than after injection of UL or C II

Region	Insulin	No of patients	3 $\bar{X}$	3 SD w	3 SD b	6 $\bar{X}$	6 SD w	6 SD b	13 $\bar{X}$	13 SD w	13 SD b	24 $\bar{X}$	24 SD w	24 SD b
Femoral	Lente	6	92	5	6	82	7	7	66	9	10	40	14	14
	Rapitard	5	85	5	5	74	5	12	56	9	23	38	12	25
Abdominal	Lente	7	88	5	6	75	6	6	50	11	16	24	10	16
	Rapitard	6	80	7	7	70	10	11	55	14	18	37	11	17

Table 4 IV Mean relative residual amounts of insulin ($\bar{X}$) at site of injection and standard deviations (SD) of repeated examinations in the same patient (w) and of single determinations (b) The values are given in percentages of the initial amount of insulin measured

Insulin	Region	No. of patients	Residual amount of insulin in percentages of injected dose (hours after injection) 1	2	4	7	15	24	35	42	40	59	66	72	T 50 per cent (hours)
Lente	Fem	6	[1]) 99 [2]) 2 9	96 4 4	89 5 6	80 6 9	60 9 8	40 9 4	28 8 8	24 7 8	21 7 2	20 5 6	18 4 7	16 4 3	20 4 4 7
	Abd	7	[1]) 98 [2]) 2 6	93 3 9	81 4 9	71 6 1	42 8 6	21 8 3	17 7 7	13 6 9	12 6 4	11 4 9	9 4 2	8 3 8	14 1 1 8
Rapitard	Fem	5	[1]) 96 [2]) 3 5	90 5.2	80 6 6	70 8 2	51 12	38 11	29 10	24 9 2	22 8 6	19 6 6	17 5 6	15 5 1	21 4 5 3
	Abd	6	[1]) 91 [2]) 2 9	86 4 4	76 5 6	67 6 9	50 9 8	37 9 4	26 8 8	21 7 8	18 7 2	20 5 6	17 4 7	14 4 3	18 9 4 1
			4 examinations per patient						3 examinations per patient			2 examinations per patient			

[1]) mean [2]) 95 per cent confidence limits

Table 4 V Mean relative residual amount of insulin at various times after subcutaneous injections of pharmacological doses of Lente and Rapitard into the femoral and abdominal regions

one site of injection, though not from the others Twenty-four hours after the injection, the residual amount of insulin varied from 42 to 56 per cent of injected dose in one patient (OB, figure 4 3), whereas a variation from 16 to 56 per cent was found in another case (ES, figure 4 3) The residual amount of insulin was up to 36 per cent of the injected dose 72 hours after the injection, but was in most cases almost negligible, when 96 hours had elapsed, in a few cases it was, however, up to 10 per cent

The absorption of Rapitard from the femoral region apparently varied less within the same patient than the absorption of Lente, but also the absorption of a single dose of Rapitard lasted for several days (figure 4 4)

In table 4 IV are given the standard deviations of single and of repeated determinations made 3, 6, 13, and 24 hours after the injection The number of examinations was too small for a further statistical treatment, the standard deviations seem, however, to be of the same order of magnitude, except for single determinations following injection of Rapitard into the femoral region In this case, the standard deviation was nearly double that of repeated examinations in the same patient The coefficient of variation corresponding to the residual amount of insulin 24 hours after injection was

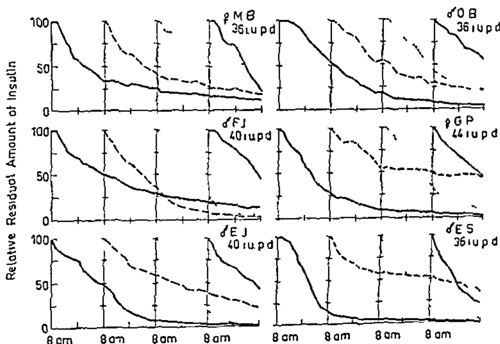


Figure 4.3 The individual absorption curves after subcutaneous injection of Lente (40 i u per ml) into the femoral region The ordinate gives the residual amount of insulin in percentages of injected dose The numbering of the 24-hour periods refers to the sites of injection into the femoral region marked in figure 4.2

Region	Insulin	No of patients	3			6			13			24		
			$\overline{X}$	SD		$\overline{X}$	SD		$\overline{X}$	SD		$\overline{X}$	SD	
				w	b		w	b		w	b		w	b
Femoral	Lente	6	92	5	6	82	7	7	66	9	10	40	14	14
	Rapitard	5	85	5	5	74	5	12	56	9	23	38	12	25
Abdominal	Lente	7	88	5	6	75	6	6	50	11	16	24	10	16
	Rapitard	6	80	7	7	70	10	11	55	14	18	37	14	17

Table 4 IV Mean relative residual amounts of insulin ($\overline{X}$) at site of injection and standard deviations (SD) of repeated examinations in the same patient (w) and of single determinations (b) *The values are given in percentages of the initial amount of insulin measured*

Insulin	Region	No of patients	Residual amount of insulin in percentages of injected dose (hours after injection)												T-50 per cent (hours)
			1	2	4	7	15	24	35	42	48	59	66	72	
Lente	Fem	6	[1]) 99 [2]) 2 9	96 4 4	89 5 6	80 6 9	60 9 8	40 9 4	28 8 8	24 7 8	21 7 2	20 5 6	18 4 7	16 4 3	20 4 4 7
	Abd	7	[1]) 98 [2]) 2 6	93 3 9	84 4 9	71 6 1	42 8 6	24 8 3	17 7 7	13 6 9	12 6 4	11 4 9	9 4 2	8 3 8	14 1 1 8
Rapitard	Fem	5	[1]) 96 [2]) 3 5	90 5 2	80 6 6	70 8 2	51 12	38 11	29 10	24 9 2	22 8 6	19 6 6	17 5 6	15 5 1	21 4 5 3
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[1]) mean [2]) 95 per cent confidence limits			4 examinations per patient						3 examinations per patient			2 examinations per patient			

Table 4 V Mean relative residual amount of insulin at various times after subcutaneous injections of pharmacological doses of Lente and Rapitard into the femoral and abdominal regions

one site of injection, though not from the others Twenty four hours after the injection, the residual amount of insulin varied from 42 to 56 per cent of injected dose in one patient (OB, figure 4 3), whereas a variation from 16 to 56 per cent was found in another case (ES, figure 4 3) The residual amount of insulin was up to 36 per cent of the injected dose 72 hours after the injection, but was in most cases almost negligible, when 96 hours had elapsed, in a few cases it was, however, up to 10 per cent

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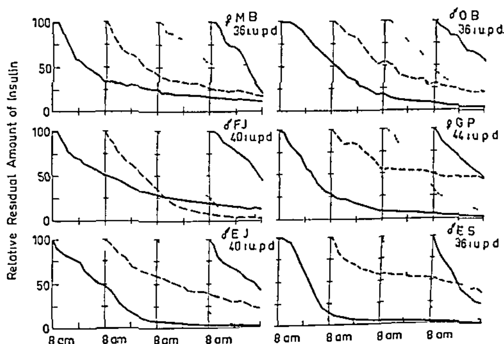


Figure 4 3 The individual absorption curves after subcutaneous injection of Lente (40 i u per ml) into the femoral region The ordinate gives the residual amount of insulin in percentages of injected dose The numbering of the 24-hour periods refers to the sites of injection into the femoral region marked in figure 4 2

From both regions, the absorption curve of Rapitard was characterized by an initial rapid component where the half time from the femoral region was about 4 hours and that from the abdominal region about $2^1/_2$ hours The half time of the slow component was about 24 hours for both regions

T-50 per cent averaged about 20 hours after injections of Lente and Rapitard into the femoral region and of Rapitard into the abdominal region A significant difference was, however, found when Lente was injected into the abdominal region, T-50 per cent then averaging 14 hours

From the said absorption determinations, the amount of insulin absorbed per day was calculated for each of the four days in each period The results are presented in figure 4 7 During the 96 hours, the average amount absorbed per day increased from approximately 60 to approximately 100 per cent of the daily injected amount of insulin

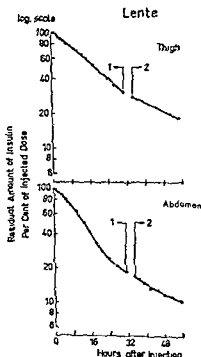


Figure 4.5 The average absorption curves after subcutaneous injection of Lente into the femoral region (6 patients) and into the abdominal one (7 patients) The mean values for the first 31 hours after injection are based on 4 examinations per patient (until 1 on the Figures From 33 to 53 hours after injection the results originate in 3 examinations per patient (from 2 on the Figures)

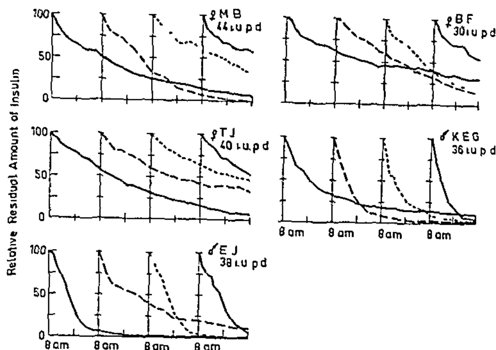


Figure 4 4 The individual absorption curves after subcutaneous injection of Rapitard (40 *i u per ml*) into the femoral region The ordinate gives the residual amount of insulin in percentages of injected dose The numbering of the 24-hour periods refers to the sites of injection into the femoral region, marked in figure 4 2

calculated for each period and for each patient and averaged 44 per cent (7–119 per cent). In the individual patient, the coefficient of variation might differ from period to period with regard to order of magnitude

In table 4 V are listed the average residual amounts of insulin at the sites of injection at various times after the injection. The values for the first 31 hours were calculated on the basis of four absorption curves for each patient, those for the interval 35 to 55 hours after injection on three curves, and those for 59 to 79 hours on two curves for each patient. The mean values for T-50 per cent are also stated in the table All values are given with 95 per cent confidence limits Figures 4 5 and 4 6 are graphs of the average courses

None of the absorption curves had a mono-exponential course Following injections of Lente, the relative absorption rate increased slowly to fall to an apparently constant value after some time The greater absorption rate for Lente during the first 24 hours was found after injection into the abdominal region The residual amounts did, however, not differ significantly until 15 hours after the injection

From both regions, the absorption curve of Rapitard was characterized by an initial rapid component where the half time from the femoral region was about 4 hours and that from the abdominal region about $2^1/_2$ hours The half time of the slow component was about 24 hours for both regions

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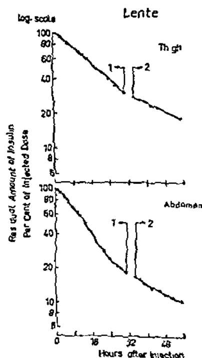


Figure 4.5 The average absorption curves after subcutaneous *injection* of *Lente* into the femoral region (6 patients) and into the abdominal *one (7 patients)* *The* mean values for the first 31 hours after injection are based on 4 examinations per patient (until 1 on the figures From 33 to 53 hours after injection the results *originate in 3* examinations per patient (from 2 on the figures)

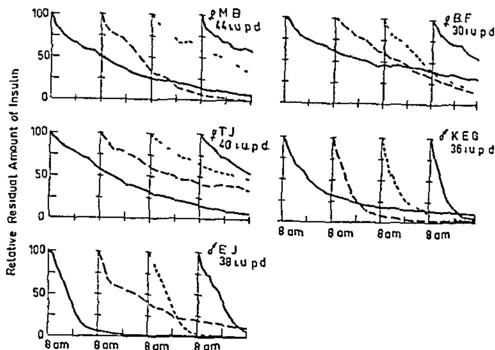


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Lente, thigh

Rapitard, thigh

Lente, abdomen

Rapitard, abdomen

Per Cent of Daily Dose

120

40

1 2 3 4

Day of Injection

Figure 4 7 Total amount of insulin absorbed per day calculated from the absorption curves for daily injections of the same insulin during 96 hours The thin lines represent the individual examinations, the fat ones the mean of the group

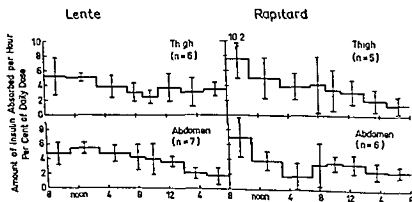


Figure 4.8 Mean relative absorption rates in the last 24 hours of 96 during which the daily dose and the preparation were unchanged When calculating the absorption rate, the amounts of insulin absorbed in each period from all four sites of injection were considered Confidence limits 95 per cent

The mean absorption rates during the last 24 hours are shown in figure 4 8 The absorptions from all four sites of injection are considered The absorption rate is given in percentages of the daily injected dose with 95 per cent confidence limits The mean absorption rate varied little after injections of Lente into the femoral region Following injections of Rapitard, the mean absorption rate was greater during the first 3-hour period after the injection than during the rest of the 24 hours The difference was, however, not significant.

Discussion

The absorption of the amorphous insulin Semilente differed on several points from those of the crystalline preparations Thus, the relative absorp-

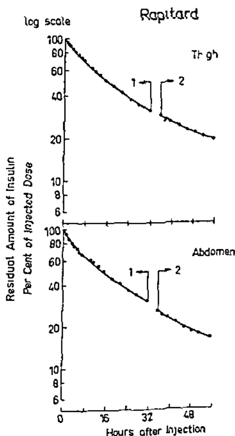


Figure 4 6 The average absorption curves after subcutaneous injection of Rapitard into the femoral region (5 patients) and into the abdominal one (6 patients) The mean values for the first 31 hours after injection are based on 4 examinations per patient (until 1 on the figures) From 33 to 53 hours after injection, the results originate in 3 examinations per patient (from 2 on the figures)

Lente, thigh

Rapitard, thigh

Lente, abdomen

Rapitard, abdomen

Per Cent of Daily Dose

120

40

1 2 3 4

Day of Injection

Figure 4.7 Total amount of insulin absorbed per day calculated from the absorption curves for daily injections of the same insulin during 96 hours. The thin lines represent the individual examinations, the fat ones the mean of the group

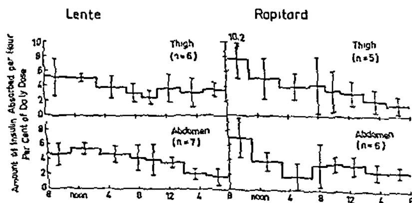


Figure 4.8 Mean relative absorption rates in the last 24 hours of 96 during which the daily dose and the preparation were unchanged. When calculating the *absorption* rate, the amounts of insulin absorbed in each period from all four sites of injection were considered. Confidence limits 95 per cent.

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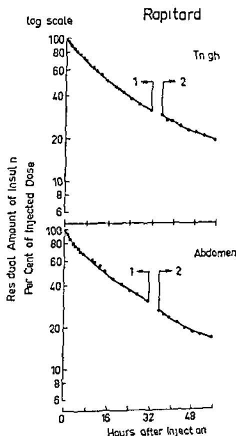


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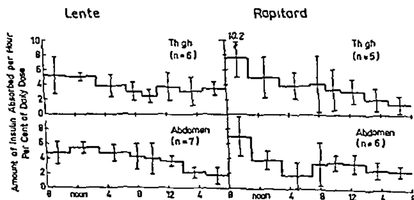


Figure 4.8 Mean relative absorption rates in the last 24 hours of 96 during which the daily dose and the preparation were unchanged. When calculating the absorption rate, the amounts of insulin absorbed in each period from all four sites of injection were considered. Confidence limits 95 per cent.

tion rate for Semilente rose slowly to a constant value This characteristic course was found for the individual as well as for the average absorption curve, and is similar to that of the dissolved insulins

Judged from the average curves, the crystalline preparations Ultralente and C II had, on the other hand, a constant relative absorption rate, if the initial part of the absorption curve for C II was neglected (see below) The absorptions of the crystalline preparations, however, varied considerably more than that of Semilente Thus, at an average residual amount of 50 per cent, the coefficient of variation was 45–73 per cent in the case of UL, 35 per cent for C II, and 19 per cent for SL (tables 4 I–III)

The absorption of the crystalline preparations sometimes stopped for several hours This phenomenon did not occur after injections of Semilente or the soluble preparations, though in a few cases after injection of the latter, the residual amounts were unchanged at two hourly determinations

From the differences demonstrated it is reasonable to assume that Semilente and the crystalline preparations are absorbed in different ways

It was shown in chapter 2 that, at any rate partially, Semilente and C II are absorbed directly to the blood (figures 2 3 and 2 4) Insulin thus can be dissolved at the site of injection The difference found between the absorption curves of the amorphous and the crystalline preparations may be explained by different rates of dissolution, in contrast to the crystalline insulin, the amorphous preparation may be so rapidly dissolved that the absorption is dependent on only the absorbing capillary surface and the molecular size of the dissolved insulin This assertion was, however, not substantiated, nor was it proved that for the crystalline preparations, the absorption curve was conditioned by the rate of dissolution Direct examination of the state of the insulin during absorption is necessary for solving these problems

The significance of insulin absorption by the lymph could not be accounted for either The question could be solved by a quantitative determination of the insulin removed from the injection region by the blood

The average absorption curve for C II had a rapid initial component, corresponding to the absorption of approximately 5 per cent of the injected dose of insulin (figure 4 1) Since the beef insulin crystals are not completely insoluble, a suspension of these crystals will contain a certain amount of dissolved insulin Schlichtkrull (in press) found this amount to be approximately 5 per cent It must therefore be assumed that the fast component of the absorption curve corresponds to the absorption of the dissolved insulin in the preparation

As mentioned before, Lente is composed of Ultralente and Semilente (70 and 30 per cent, respectively), Rapitard of C II and Actrapid (75 and

2a per cent, respectively) As also the absorption from the femoral region was examined for each of these components, a calculation could be made of the absorptions from this region of the two preparations The results of this calculation are presented in table 4 VI The determined and the calculated

		Amount of insulin in percentages of injected dose (hours after injection)							
		1	2	3	4	7	8	24	27
Lente	Deter minations	[1]) 99 [2]) 2 9	96 4 4	92 5 1	89 5 6	80 6 9	– –	40 9 4	35 3 3
	Calcula tions	[1]) 98 [2]) 1 2	97 1 6	94 1 6	91 2 1	– –	79 3 6	49 13	45 14
Rapitard	Deter minations	[1]) 96 [2]) 3 5	90 5 2	85 6 0	80 6 6	70 8.2	– –	38 11	35 11
	Calcula tions	[1]) 95 [2]) 1 3	86 2 3	80 3 1	76 2 9	65 5 8	– –	40 6 9	37 12

[1]) mean [2]) 95 per cent confidence limits

Table 4 VI Comparison between the residual amounts of insulin found by the actual determinations and the corresponding calculated values. The calculations were based on the absorption curves determined for the individual components of Lente and Rapitard

absorption courses of the two preparations did not differ significantly In spite of the size of the experimental error, the results seem to indicate that the individual components of Lente and Rapitard have preserved their characteristic absorptions

Like the dissolved insulins, Lente was more rapidly absorbed from the abdominal region than from the femoral one As for Rapitard, the absorptions from the two regions did not differ The regional difference in the absorption of Lente might be due to the fact that the amorphous component – like the dissolved insulin – had a faster absorption from the femoral region, and that the crystalline component was absorbed in the same way from the two regions

When approximately 16 hours had elapsed, the absorption of Rapitard had a simple mono-exponential course that did not differ between the two regions. The half time for this part of the curve was about 26 hours This suggests that the absorption of the crystalline component is widely independent of the local blood flow

The clinical importance of the results is that the much recommended change of region for insulin injections may cause an undesirable variation

tion rate for Semilente rose slowly to a constant value This characteristic course was found for the individual as well as for the average absorption curve, and is similar to that of the dissolved insulins

Judged from the average curves, the crystalline preparations Ultralente and C II had, on the other hand, a constant relative absorption rate, if the *initial part of the absorption curve* for *C II* was neglected (see below) The absorptions of the crystalline preparations, however, varied considerably more than that of Semilente Thus, at an average residual amount of 50 per cent, the coefficient of variation was 45–73 per cent in the case of UL, 35 per cent for C II, and 19 per cent for SL (tables 4 I–III)

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From the differences demonstrated it is reasonable to assume that Semilente and the crystalline preparations are absorbed in different ways

It was shown in chapter 2 that, at any rate partially, Semilente and C II are absorbed directly to the blood (figures 2 3 and 2 4) Insulin thus can be dissolved at the site of injection The difference found between the absorption curves of the amorphous and the crystalline preparations may be explained by different rates of dissolution, in contrast to the crystalline insulin, the amorphous preparation may be so rapidly dissolved that the absorption is dependent on only the absorbing capillary surface and the molecular size of the dissolved insulin This assertion was, however, not substantiated, nor was it proved that for the crystalline preparations, the absorption curve was conditioned by the rate of dissolution Direct examination of the state of the insulin during absorption is necessary for solving these problems

The significance of insulin absorption by the lymph could not be accounted for either The question could be solved by a quantitative determination of the insulin removed from the injection region by the blood

The average absorption curve for C II had a rapid initial component corresponding to the absorption of approximately 5 per cent of the injected dose of insulin (figure 4 1) Since the beef insulin crystals are not completely insoluble, a suspension of these crystals will contain a certain amount of dissolved insulin Schlichtkrull (in press) found this amount to be approximately 5 per cent It must therefore be assumed that the fast component of the absorption curve corresponds to the absorption of the dissolved insulin in the preparation

As mentioned before, Lente is composed of Ultralente and Semilente (70 and 30 per cent, respectively), Rapitard of C II and Actrapid (75 and

25 per cent, respectively) As also the absorption from the femoral region was examined for each of these components, a calculation could be made of the absorptions from this region of the two preparations The results of this calculation are presented in table 4 VI The determined and the calculated

		Amount of insulin in percentages of injected dose (hours after injection)							
		1	2	3	4	7	8	24	27
Lente	Determinations	[1]) 99	96	92	89	80	–	40	35
		[2]) 2 9	4 4	5 1	5 6	6 9	–	9 4	3 3
	Calculations	[1]) 98	97	94	91	–	79	49	45
		[2]) 1 2	1 6	1 6	2 1	–	3 6	13	14
Rapitard	Determinations	[1]) 96	90	85	80	70	–	38	35
		[2]) 3.5	5.2	6 0	6 6	8 2	–	11	11
	Calculations	[1]) 95	86	80	76	65	–	40	37
		[2]) 1 3	2.3	3 1	2 9	5 8	–	6 9	12

[1]) mean [2]) 95 per cent confidence limits

Table 4 VI Comparison between the residual amounts of insulin found by the actual determinations and the corresponding calculated values. The calculations were based on the absorption curves determined for the individual components of Lente and Rapitard

absorption courses of the two preparations did not differ significantly In spite of the size of the experimental error, the results seem to indicate that the individual components of Lente and Rapitard have preserved their characteristic absorptions

Like the dissolved insulins, Lente was more rapidly absorbed from the abdominal region than from the femoral one As for Rapitard, the absorptions from the two regions did not differ The regional difference in the absorption of Lente might be due to the fact that the amorphous component – like the dissolved insulin – had a faster absorption from the femoral region, and that the crystalline component was absorbed in the same way from the two regions.

When approximately 16 hours had elapsed, the absorption of Rapitard had a simple mono-exponential course that did not differ between the two regions The half time for this part of the curve was about 26 hours This suggests that the absorption of the crystalline component is widely independent of the local blood flow

The clinical importance of the results is that the much recommended change of region for insulin injections may cause an undesirable variation

tion rate for Semilente rose slowly to a constant value This characteristic course was found for the individual as well as for the average absorption curve, and is similar to that of the dissolved insulins

Judged from the average curves, the crystalline preparations Ultralente and C II had, on the other hand, a constant relative absorption rate, if the initial part of the absorption curve for C II was neglected (see below) The absorptions of the crystalline preparations, however, varied considerably more than that of Semilente Thus, at an average residual amount of 50 per cent, the coefficient of variation was 45–73 per cent in the case of UL, 35 per cent for C II, and 19 per cent for SL (tables 4 I–III)

The absorption of the crystalline preparations sometimes stopped for several hours This phenomenon did not occur after injections of Semilente or the soluble preparations, though in a few cases after injection of the latter, the residual amounts were unchanged at two hourly determinations

From the differences demonstrated it is reasonable to assume that Semilente and the crystalline preparations are absorbed in different ways

It was shown in chapter 2 that, at any rate partially, Semilente and C II are absorbed directly to the blood (figures 2 3 and 2 4) Insulin thus can be dissolved at the site of injection The difference found between the absorption curves of the amorphous and the crystalline preparations may be explained by different rates of dissolution, in contrast to the crystalline insulin, the amorphous preparation may be so rapidly dissolved that the absorption is dependent on only the absorbing capillary surface and the molecular size of the dissolved insulin This assertion was, however, not substantiated, nor was it proved that for the crystalline preparations, the absorption curve was conditioned by the rate of dissolution Direct examination of the state of the insulin during absorption is necessary for solving these problems

The significance of insulin absorption by the lymph could not be accounted for either The question could be solved by a quantitative determination of the insulin removed from the injection region by the blood

The average absorption curve for C II had a rapid initial component, corresponding to the absorption of approximately 5 per cent of the injected dose of insulin (figure 4 1) Since the beef insulin crystals are not completely insoluble, a suspension of these crystals will contain a certain amount of dissolved insulin Schlichtkrull (in press) found this amount to be approximately 5 per cent It must therefore be assumed that the fast component of the absorption curve corresponds to the absorption of the dissolved insulin in the preparation

As mentioned before, Lente is composed of Ultralente and Semilente (70 and 30 per cent, respectively), Rapitard of C II and Actrapid (75 and

Chapter 5

ABSORPTION AND BLOOD SUGAR CONCENTRATION

Dissolved insulin

In 118 patients, confined to bed, Binder et al. (1967) determined the course of the blood sugar concentration concurrently with that of the absorption. The investigations were carried out between 8 and 16. During these eight hours the patients were given: At 8: 50 g bread, 10 g fat, and 150 g milk. At 12: 80 g bread, 20 g fat, one egg, and 200 g milk. At 15: 30 g bread, 5 g fat, and 15 g cream. Insulin was injected immediately before breakfast. The postprandial rise in blood sugar concentration occurred one hour after the injection (figure 5.1.). When SI was injected into the femoral region, the rise averaged 0.87 g per litre. The corresponding relative amount of absorbed insulin was 9 per cent. During the first hour after injection of A into the abdominal region, the average amount of absorbed insulin was 32 per cent of the injected dose. This corresponds to a rise in blood sugar concentration of not more than 0.38 g per litre. The rise in blood sugar concentration was steeper where the absorption was complete than in those cases where it lasted longer than the examination period. Of the 118 patients, 33 showed hypoglycemic reactions that required prompt administration of carbohydrate. Immediately before this was given, capillary blood was sampled for determination of the blood sugar concentration. This was in all 33 cases below 0.60 g per litre. The reactions occurred most often in patients with a high absorption rate.

Owing to the additional administration of carbohydrate, these 33 patients did not fulfil the standard conditions with regard to diet laid down for the examination; they, consequently, were disregarded in the subsequent calculations.

The examination lasted for 8 hours and fell into 3 periods. The first lasted from 8 to 9 (0–1) and corresponded to the said postprandial rise in blood sugar concentration, the second from 9 to 12 (1–4), and the third from 12 to 16 (4–8). The correlation between the absorbed amount of insulin and

in the amount of insulin absorbed per day It should therefore be recommended that injections are given into the same region for a fairly long period When changing to another region, patients should be more frequently controlled until the adjustment is re-established

Nora, Smith, and Cameron (1964) introduced ^{131}I-insulin in acid solution into ampoules containing Lente The next day the mixture was injected subcutaneously and the absorption examined When insulin in acid solution is added to Lente (Schlichtkrull, in press), the action of the former is delayed, *whereas there is no change in the characteristic action of the crystalline* component. The results of Nora et al , therefore cannot be taken for representative of the absorption of Lente

When crystalline preparations are injected regularly once or twice a day, the fact that their absorptions last essentially more than 24 hours causes an accumulation of insulin in subcutis The duration of absorption naturally determines the time for equilibration between the amounts injected and absorbed per 24 hours In the present investigation, an average of 96 hours elapsed before equilibrium was reached This finding confirms the clinical observation that a given adjustment with a protracted acting insulin cannot be evaluated until the same dose has been administered for several days

As the absorption varies much – also in the individual patient – a large number of determinations are necessary for characterizing the absorption course of a given insulin preparation The present method therefore will hardly be included among those employed for determining the optimal insulin dose to a given patient The results showed, on the other hand, that a direct examination of the absorption properties of a preparation may supply information otherwise unattainable

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For each period, the absorbed amount of insulin (ABS INS) in international units, and the variation in blood sugar concentration (ΔBS) were calculated It was to be expected that the patients differed with regard to basic insulin requirement and sensitivity to a given amount of insulin absorbed The statistical treatment of the material consequently had to include analyses of correlation The coefficients of correlation within patients were calculated from the following expression

$$r_w = \frac{\Sigma\Sigma\,(y_{ij} - y_i)\,(x_{ij} - \bar{x}_i)}{\sqrt{\Sigma\Sigma\,(y_{ij} - y_i)^2\;\Sigma\Sigma\,(x_{ij} - x_i)^2}} \quad (1)$$

where x_{ij} and y_{ij} denote ABS INS and ΔBS, respectively, calculated from two successive determinations in the i th patient, x_i and y_i are the corresponding mean values of the determinations in the i th patient

The coefficients of correlation between patients were calculated from

$$r_b = \frac{\Sigma\,(y_i - \bar{\bar{y}})\,(x_i - x)}{\sqrt{\Sigma\,(y_i - y)^2\,\Sigma\,(x_i - x)^2}} \quad (2)$$

where x and $\bar{\bar{y}}$ are the total mean values

At the above mentioned analysis of correlation, the following regression equation was employed

$$\Delta BS = a + b\,(ABS\ INS) \quad (3)$$

Another regression equation applicable to the analysis is

$$\Delta BS = a + b_1\,(ABS\ INS)_{1-3} + b_2\,(ABS\ INS)_4 \quad (4)$$

Correlation between percentage of insulin absorbed and	Number of examinations	Coefficient of correla ion	P-value
rise in blood sugar concentration 0 hour–1 hour	85	−0.379	< 0 001
fall in blood sugar concentration 1 hour–4 hrs	85	0 307	< 0 01
rise in blood sugar concentration 4 hrs.–8 hrs.	85	−0 541	< 0 001

Table 5 I Correlation between the relative amount of absorbed insulin and change in blood sugar concentration in 85 patients all confined to bed. The dose was in all cases 12 i.u. of dissolved insulin injected subcutaneously No other insulin was given during the examination period.

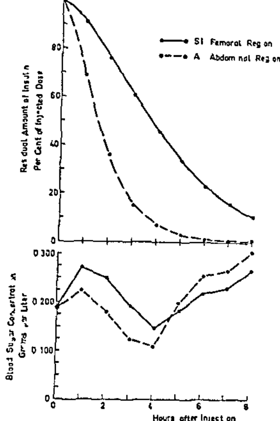


Figure 5 1 Average curves of absorption and blood sugar concentration after subcutaneous injection of 0 3 ml (12 i u) of, respectively, Insulin Novo (SI) and Actrapid (A) into the femoral and the abdominal region

the corresponding variation in blood sugar concentration was calculated for each period

The result is given in table 5 I It should be noted that for the first and the third period, the calculation is based on the rise in blood sugar concentration and for the second period, on the fall in blood sugar concentration Though the coefficient of correlation was never especially high, a significant correlation was found for all periods

Lente and Rapitard

The fact that the absorptions of Lente and Rapitard may last longer than 24 hours gives rise to the question whether the blood sugar concentration during a given 24-hour period is correlated to the amount of insulin absorbed from depots injected prior to the period

Simultaneous determinations of the residual amount of insulin and of the blood sugar concentration were performed at 8, 11, 15, 21, and 2 The examination was thereby divided into 5 periods for every 24 hours

For each period, the absorbed amount of insulin (ABS INS) in international units, and the variation in blood sugar concentration (ΔBS) were calculated It was to be expected that the patients differed with regard to basic insulin requirement and sensitivity to a given amount of insulin absorbed. The statistical treatment of the material consequently had to include analyses of correlation The coefficients of correlation within patients were calculated from the following expression

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where x_{ij} and y_{ij} denote ABS INS and ΔBS, respectively, calculated from two successive determinations in the i-th patient, $\bar{x}_i$ and $\bar{y}_i$ are the corresponding mean values of the determinations in the i th patient

The coefficients of correlation between patients were calculated from

$$r_b = \frac{\Sigma\,(\bar{y}_i - y)\,(x_i - \bar{\bar{x}})}{\sqrt{\Sigma\,(\bar{y}_i - y)^2\;\Sigma\,(x_i - x)^2}} \tag{2}$$

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At the above-mentioned analysis of correlation, the following regression equation was employed

$$\Delta BS = a + b\,(ABS\ INS) \tag{3}$$

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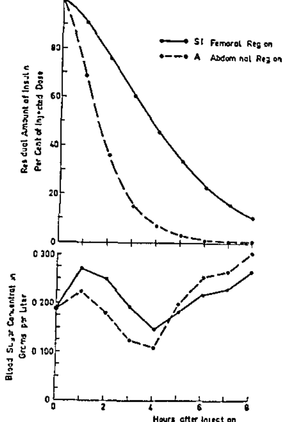


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Simultaneous determinations of the residual amount of insulin and of the blood sugar concentration were performed at 8, 11, 15, 21, and 2 The examination was thereby divided into 5 periods for every 24 hours

Period	Δ BS (mg per 100 ml per hour)	ABS.INS. (i.u. per hour)	r_w	r_b
8–11	−0 7	2 19	0 531	−0 087
11 15	−9 1	1.80	0 672*	0 180
15–21	0.7	1 43	0 734*	0 088
21– 2	7 8	1 39	−0 308	0.300
2 8	−1 0	0 91	0.243	−0.235

*) P < 0 01

Table 5 V Correlation between blood sugar concentrat on during the fourth 24-hour period and amount of insulin absorbed from all the 4 depots injected

where $(ABS\ INS)_{1\ 3}$ denotes the amount of insulin absorbed from the 3 previously injected depots, and $(ABS\ INS)_4$ the amount of insulin absorbed from the latest injected depot, b_1 and b_2 are the absorption rates on the fourth day from, respectively, the three previous sites of injection and the fourth It appears that if $b_1=b_2$, formula 4 reduces to formula 3 By applying multiple analyses of correlation, it can be examined whether $(ABS\ INS)_{1\ 3}$ as well as $(ABS\ INS)_4$ gives statistically significant reductions of the variations in ΔBS If, on examination, b_1 and b_2 prove identical, then formulas 3 and 4 are equally apt expressions of the absorption

In the tables 5 II V are presented the results of the simple analyses of correlation carried out for each of the four consecutive days when the labelled insulin was injected

On the first day, a significant correlation was found in the periods 8–15 ($P < 0\ 05$) and 15–21 ($P < 0\ 01$) No significant correlation was found in any period during the next 24 hours On the third day, ΔBS and $(ABS\ INS)_{1\ 3}$ were significantly correlated in the period 2–8 ($P < 0\ 05$) A significant correlation ($P < 0\ 01$) was found on the fourth day in the periods 11 15 and 15–21 It should be noted that in the period 21–2, r_w seems to be smaller than in the other periods There was no demonstrable rise in r_w during the 96 hours

The coefficients of the correlation within patients are shown in figure 5 2

Table 5 VI shows the result of a simple correlation analysis between ΔBS (4 day) and $(ABS\ INS)_{1-3}$ The parameters were not significantly correlated in any period

Table 5 VII illustrates the simple correlations between ΔBS (4 day) and $(ABS\ INS)_4$ In this case, the parameters were significantly correlated in the first 3 periods

Period	A BS (mg per 100 ml per hour)	ABS INS (i u per hour)	r_w	r_b
8–11	2 5	1 63	0 621 *	0 457
11–15	−10 4	1 30	0 437	0 332
15–21	−2 4	1 04	0 822**	0 743**
21– 2	8 7	0 96	0 217	0 102
2– 8	0 1	0 55	0 256	0 371

* P < 0 05 ** P < 0 01

Table 5 II Correlation between blood sugar concentration during the first 24 hours and amount of insulin absorbed from the depot injected on the same day, r_w and r_b are the coefficients of correlation within patients (r_w) and between patients (r_b)

Period	ABS (mg per 100 ml per hour)	ABS INS (i u per hour)	r_w	r_b
8–11	2 9	2 14	0 389	0 339
11–15	−7 7	1 76	0 522	0 691*
15–21	−4 8	1 00	0 352	−0 045
21– 2	7 6	1 13	−0 299	0 477
2– 8	0 8	0 75	0 444	0 211

*) P < 0 05

Table 5 III Correlation between blood sugar concentration during the second 24-hour period and amount of insulin absorbed from the depots injected on the first and the second day

Period	ABS (mg per 100 ml per hour)	ABS INS (i u per hour)	r_w	r_b
8–11	−2 4	2 39	0 161	0 292
11–15	−3 7	1 80	0 479	0 218
15–21	4 5	1 60	0 345	0 186
21– 2	9 3	1 23	−0 333	0 532
2– 8	−3 0	1 00	0 626*	0 616*

*) P < 0 05

Table 5 IV Correlation between blood sugar concentration during the third 24-hour period and amount of insulin absorbed from the depots injected on the first 3 days

Period	Δ BS (mg per 100 ml per hour)	ABS.INS. (μ.u. per hour)	r_w	r_b
8–11	−0 7	2 19	0 531	−0.087
11 15	−9 1	1 80	0 672*	0 180
15–21	0 7	1 43	0 734*	0 088
21– 2	7 8	1 39	−0 308	0.300
2– 8	−1 0	0 91	0.243	−0.235

*) P < 0 01

Table 5.V Correlation between blood sugar concentration during the fourth 24-hour period and amount of insulin absorbed from all the 4 depots injected.

where $(\text{ABS INS})_{1-3}$ denotes the amount of insulin absorbed from the 3 previously injected depots, and $(\text{ABS INS})_4$ the amount of insulin absorbed from the latest injected depot, b_1 and b_2 are the absorption rates on the fourth day from, respectively, the three previous sites of injection and the fourth It appears that if $b_1=b_2$, formula 4 reduces to formula 3 By applying multiple analyses of correlation, it can be examined whether $(\text{ABS INS})_{1-3}$ as well as $(\text{ABS INS})_4$ gives statistically significant reductions of the variations in ΔBS If, on examination, b_1 and b_2 prove identical, then formulas 3 and 4 are equally apt expressions of the absorption

In the tables 5 II–V are presented the results of the simple analyses of correlation carried out for each of the four consecutive days when the labelled insulin was injected

On the first day, a significant correlation was found in the periods 8–15 ($P < 0.05$) and 15–21 ($P < 0.01$) No significant correlation was found in any period during the next 24 hours. On the third day, ΔBS and $(\text{ABS INS})_{1-3}$ were significantly correlated in the period 2–8 ($P < 0.05$). A significant correlation ($P < 0.01$) was found on the fourth day in the periods 11–15 and 15–21 It should be noted that in the period 21–2, r_w seems to be smaller than in the other periods There was no demonstrable rise in r_w during the 96 hours

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8–11	2 5	1 63	0 621 *	0 457
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2– 8	0 1	0 55	0 256	0 371

* P < 0 05 ** P < 0 01

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8–11	2 9	2 14	0 389	0 339
11–15	–7 7	1 76	0 522	0 691*
15–21	–4 8	1 00	0 352	–0 045
21– 2	7 6	1 13	–0 299	0 477
2– 8	0 8	0 75	0 444	0 211

*) P < 0 05

Table 5 III Correlation between blood sugar concentration during the second 24 hour period and amount of insulin absorbed from the depots injected on the first and the second day

Period	ΔBS (mg per 100 ml per hour)	ABS INS (i u per hour)	r_w	r_b
8–11	–2 4	2 39	0 161	0 292
11–15	–3 7	1 80	0 479	0 218
15–21	4 5	1 60	0 345	0 186
21– 2	9 3	1 23	–0 333	0 532
2– 8	–3 0	1 00	0 626*	0 646*

*) P < 0 05

Table 5 IV Correlation between blood sugar concentration during the third 24-hour period and amount of insulin absorbed from the depots injected on the first 3 days.

Period	ΔBS_4 (mg per 100 ml per hour)	$ABS.INS._4$ (μU per hour)	r_w	$\alpha \pm SD$	
8-11	−0.7	1.56	0.607*	13.5	5.4
11-15	−9.1	1.35	0.752**	18.9	5.0
15-21	0.7	1.00	0.798**	20.5	4.7
21-2	7.8	1.02	−0.329	−8.0	6.9
2-8	−1.0	0.64	0.232	9.8	18.0

*) $P < 0.05$ **) $P < 0.01$

Table 5.VII Correlation between the fall in blood sugar concentration on the fourth 24-hour period $(\Delta BS)_4$ and the absorption rate during the same interval. The calculation of the absorption rate was based on the absorptions of the insulin injected on the 3 preceding days $(ABS\ INS)_{1-3}$ and of that injected on the same day $(ABS\ INS)_4$, r_w denotes the correlation coefficient and α the slope of the regression line, SD is the standard deviation of the latter parameter

Period	b_1	b_4	R	F-test for $ABS.INS._{1-3}$	$ABS.INS._4$
8-11	− 2.5	13.3	0.608	0.02	5.32*
11-15	3.2	20.1	0.752*	0.00	7.22*
15-21	−10.6	19.5	0.805**	0.32	14.5**
21-2	− 2.8	− 8.8	0.322	0.99	0.02
2-8	5.9	9.0	0.245	0.45	0.07

*) $P < 0.05$ *) $P < 0.01$

Table 5.VIII Partial and multiple correlation coefficients for the blood sugar concentration during the fourth 24-hour period correlated to the absorption rate from the 3 previous sites of injection (b_1) as well as to that from the fourth site of injection (b_4), R is the multiple correlation coefficient.

Discussion

In diabetic patients treated optimally with diet and one or two daily injections of insulin, the diurnal blood sugar concentration curve varies considerably In some, the movements are but small and within the range where no hypoglycemic reactions and glucosuria occur, in others, the concentration varies from hypoglycemic to hyperglycemic values Besides the variation between patients, there is a variation within patients in the reproducibility of the diurnal curve

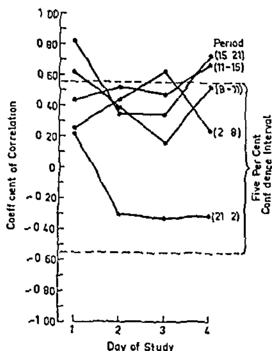


Figure 5 2 Variation within patients in the coefficients of correlation between absorbed insulin and blood sugar concentration

The results of the multiple regression analyses are given in table 5 VIII Also from this analysis, the only significant correlation discernible was between (ABS INS)$_4$ and $\triangle$BS (4 day) Owing to the great standard deviations of the calculated regression coefficients, the probability of obtaining statistically significant regression coefficients is very small in a material of this size

Period	Δ BS$_4$ (mg per 100 ml per hour)	ABS INS$_{1-4}$ (i u per hour)	r_w	$\alpha \pm$ SD	
8–11	–0 7	0 63	–0 185	– 9 6	15 4
11–15	–9 1	0 46	–0 502	–24 0	12 5
15–21	0 7	0 43	–0 373	–33 4	25 1
21– 2	7 8	0 38	0 149	7 0	14 1
2– 8	–1 0	0 28	0 134	9 8	21 9

Table 5 VI

Period	ΔBS$_4$ (mg per 100 ml per hour)	ABS INS$_4$ (i.u. per hour)	r_w	α ± SD	
8-11	−0 7	1 56	0 607*	13 5	5 4
11-15	−9 1	1.35	0 752**	18 9	5 0
15-21	0 7	1 00	0 798**	20.5	4 7
21- 2	7 8	1 02	−0 329	− 8 0	6 9
2 8	−1 0	0 64	0 232	9 8	18 0

*) $P < 0.05$ **) $P < 0.01$

Table 5.VI-VII Correlation between the fall in blood sugar concentration on the fourth 24-hour period (ΔBS)$_4$ and the absorption rate during the same interval The calculation of the absorption rate was based on the absorptions of the insulin injected on the 3 preceding days (ABS.INS)$_{1-3}$ and of that injected on the same day (ABS INS)$_4$, r_w denotes the correlation coefficient and α the slope of the regression line, SD is the standard deviation of the latter parameter

Period	b_1	b_2	R	F-test for	
				ABS.INS.$_{1-3}$	ABS.INS.$_4$
8-11	− 2 5	13 3	0 608	0 02	5.32*
11 15	3 2	20 1	0 752*	0 00	7 22*
15-21	−10 6	19 5	0 805**	0 32	14 5**
21- 2	− 2 8	− 8 8	0 322	0.99	0 02
2- 8	5 9	9 0	0 245	0 45	0 07

*) $P < 0.05$ *) $P < 0.01$

Table 5.VIII Partial and multiple correlation coefficients for the blood sugar concentration during the fourth 24-hour period correlated to the absorption rate from the 3 previous sites *of injection* (b_1) *as well as to that from the fourth site of injection* (b_2), *R is the multiple* correlation coefficient.

Discussion

In diabetic patients treated optimally with diet and one or two daily injections of insulin, the diurnal blood sugar concentration curve varies considerably In some, the movements are but small and within the range where no hypoglycemic reactions and glucosuria occur, in others, the concentration varies from hypoglycemic to hyperglycemic values Besides the variation between patients, there is a variation within patients in the reproducibility of the diurnal curve.

The results of the present investigations showed that the latter variation is not due solely to variations in the absorption rate of insulin

Insulin is only one of the factors that determine the blood sugar concentration Owing to its hypoglycemic effect, it may induce an increased secretion of agents the actions of which are reflected by an increase in the blood sugar concentration Of these agents, catecholamines, glucocorticoids, the growth hormone, and glucagon are the most important ones Within 45 minutes after hypoglycemia had occurred, the concentrations of such agents were found to have increased (Williams and Wood 1965) The hyperglycemia thus induced will often be resistent to insulin (Pfeiffer 1965, Williams 1968). It is well-established that, besides hypoglycemia, a number of stimuli may induce an acutely increased secretion of catecholamines and glucocorticoids The healthy organism responds to the resulting rise in blood sugar concentration with an enhanced secretion of insulin from pancreas, whereas the diabetic one absorbs the injected insulin at a rate independent of the blood sugar concentration

Differences in the functional level of the thyroid gland also cause the blood sugar concentration course to vary between patients, because the insulin requirement rises with increasing activity of the thyroid gland

Sensitivity to insulin, too, varies between patients (Samols 1965) Some patients will be able to secrete a certain amount of insulin, but the capacity of the insular tissue varies (Seltzer 1961) In investigations of the blood sugar concentration course in a group of patients, this also presents a source of variation

Another source is the diurnal variation in the activity and sensitivity of the various blood sugar regulating mechanisms This is indicated by the fact that different therapies shall be employed to adjust the individual patients Some are well-controlled at one daily injection of a protracted acting preparation, in others two daily injections are necessary, while others again shall be treated once or twice a day with mixtures of protracted acting and short acting insulins

In the present work the blood sugar concentration was determined at intervals up to six hours According to the above, a rise in the blood sugar concentration found during such an interval may actually be due to the fact that, between the two determinations, a fall in the concentration to a hypoglycemic value has triggered off counter mechanisms

It was therefore to be expected that the amount of insulin absorbed during a certain interval determined only a minor part of the variation in blood sugar concentration found during the same interval

The material for the investigation of Lente and Rapitard consisted only

of patients whose diabetes was difficult to adjust The results make unlikely that the course of absorption was decisive of the lability of the individual patient s diabetes mellitus The complete lack of correlation between ΔABS INS and ΔBS during the period 21 to 2 is peculiar and cannot be accounted for

Examining the absorption of a given insulin preparation thus seems to offer no short cut to assessing the applicability of the preparation to diabetes therapy For this purpose is still demanded an exhaustive clinical testing on a large material selected at random

6*

Summary and conclusions

Chapter 1

Insulin labelled with ^{125}I was found to behave like carrier insulin when injected subcutaneously It was also found that external monitoring of the radioactivity remaining at the site of injection always provides a reliable measure of the amount of insulin remaining in the tissue

Chapter 2

It was a characteristic feature of the course of absorption for dissolved insulin that the relative absorption rate rose during the first hours after the injection, whereafter it remained constant The initial phase was dependent on the insulin concentration of the injection and on the volume of injection At low insulin concentrations and small volumes, the relative absorption rate was constant during the whole course of the absorption The literature supports the assumption that this phenomenon is due to insulin concentrations above a certain level in the tissue inducing a transient local depression of the microcirculation The significance of the volume cannot be explained The results emphasize the necessity of more detailed investigations into the effect of insulin on the microcirculation

Experimental results showed that, at any rate partially, insulin is absorbed directly from tissue to blood The direct absorption took place irrespective of whether the insulin was injected in a dissolved, amorphous or crystalline state The absorption rate for dissolved insulin injected intramuscularly was closely related to resting blood flow through the muscle concerned

Chapter 3

The pH of the solvent was found to influence the duration of the absorption in that the latter was more rapidly completed for insulin in neutral solution The regional differences between the absorptions of insulin injected subcutaneously and intramuscularly were greater in the former case The absorption curves obtained had the same shape irrespective of type of injection The probability was established that the regional differences were due to differences in blood flow

parameters examined in the patients, the absorption was significantly correlated only to the sense of vibration, as a diminishing sense of vibration was combined with a diminishing T-50 per cent Based on a postulated correlation between the sense of vibration and the capillary condition, an explanation was given of the correlation demonstrated by other investigators between increased capillary permeability and long duration of diabetes mellitus

Chapter 4

Except for its considerably longer duration, the course of absorption for an amorphous pig insulin preparation was similar to that for dissolved insulin When insulin was injected as a suspension of crystals, the absorption had, on the contrary, a course characterized by changing rates of absorption The absorption curve for crystalline insulin had in general a simple mono-exponential form with a half time between 30 and 40 hours

Two preparations in current use in the diabetes therapy contain two components For the examination of these preparations, it was attempted, as far as possible, to obtain conditions in the patients similar to those during treatment Therefore, therapeutic doses of labelled insulin were injected daily for four days The absorption rate varied considerably after each individual injection, and the results showed great variations within as well as between patients.

No certain conclusion could be reached on the absorption mechanism in the case of preparations with protracted action Presumably, insulin is dissolved in situ to be absorbed directly from tissue to blood, but there is no proof of this theory

It must be concluded that the examination is too time-consuming to be of practical use for fixing the individual patient's insulin dosage It should on the other hand be emphasized that the direct examination of the absorption properties of a preparation may give information otherwise unattainable

Chapter 5

The amount of dissolved insulin absorbed from a subcutaneous depot during a given time was significantly correlated to the changes in blood sugar concentration found during the same time The coefficient of correlation was, however, not high This was taken to mean that other factors had a regu- ... ence on the actual blood sugar concentration

f protr... te I nsulin in therapeutic doses was examined

Summary and conclusions

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Resumé

Kapitel 1

Det er vist, at insulin mærket med ^{125}I følger carrierinsulin efter subcutan injektion Det er endvidere vist, at ekstern *måling af den på injektionsstedet* til enhver tid resterende mængde radioaktivitet giver et pålideligt udtryk for den i *vævet resterende insulinmængde*

Kapitel 2

Absorptionsforløbet af opløst insulin var karakteriseret ved, at den relative *absorptionshastighed var stigende de første timer* efter injektionen for derefter at antage en konstant værdi Den initiale fase var afhængig af insulinkoncentrationen i injektatet og af injektionsvolumen Ved små insulinkoncentrationer og små volumina sås en konstant relativ absorptionshastighed under hele absorptionsforløbet Det formodes – med støtte i litteraturen – at fænomenet skyldes, at der over en vis insulinkoncentration i vævet udløstes en forbigående lokal hæmning af mikrocirkulationen Volumeneffekten henstår uforklaret Resultaterne peger på nødvendigheden af mere detaillerede undersøgelser til belysning af insulinets virkning på mikrocirkulationen

Resultatet af eksperimentelle undersøgelser viste, at insulin – i hvert fald delvis – absorberedes direkte fra væv til blod Dette fandt sted uanset om insulinet var i opløst, amorf eller krystallinsk form i injektatet Der påvistes også en nøje korrelation mellem absorptionshastigheden af opløst intramuskulært injiceret insulin og blodgennemstrømningen under hvile i den samme muskel

Kapitel 3

Absorption af opløst insulin var hurtigere afsluttet, når insulinet var opløst ved neutral reaktion Absorptionen af opløst insulin udviste regionale forskelle, der var betydelig større efter subcutan injektion Absorptionsforløbets *form var den samme efter subcutan* som efter intramuskulær injektion Det sandsynliggøres, at de påviste regionale forskelle kunne være betinget af forskelle i blodgennemstrømning

Af en række parametre hos de undersøgte patienter var absorptionen –

in a group of patients with a labile diabetes Also these results showed that the amount of insulin supplied during a given time determined only a minor part of the variation in blood sugar concentration found during the same time

Thus, examining the conditions of absorption for a given insulin preparation apparently offers no short cut to an estimation of the suitability of the preparation for treatment of diabetes mellitus

et givet tidsrum tilførte insulinmængde kun var bestemmende for en mindre del af den i samme tidsrum fundne variation i blodsukkerkoncentrationen

En undersøgelse af et givet insulinpræparats absorptionsforhold synes således ikke at byde på genveje til vurderingen af dette præparats egnethed i terapien af diabetes mellitus

udtrykt ved den tid det varede indtil de første 50 % af det injicerede insulin

tionssansen, idet en afta-

T-50 % Udfra en postu-
leret sammenhæng mellem vibrationssans og kapillærernes tilstand forklares sammenhængen ved den af andre påviste øgede kapillærpermeabilitet hos patienter med langvarig diabetes mellitus

Kapitel 4.

Absorptionen af et amorft svineinsulinpræparat havde samme forløb som absorptionen af opløst insulin, idet absorptionen dog strakte sig over væsentlig længere tid Dette i modsætning til absorptionen af insulin injiceret i krystallinsk form, hvor forløbet efter den enkelte injektion var karakteriseret af vekslende absorptionshastigheder I gennemsnit fulgte absorptionen af insulinkrystallerne en simpel mono-eksponentiel form med en halveringstid, der var mellem 30 og 40 timer

Ved undersøgelsen af to sammensatte præparater, der finder hyppig anvendelse i behandlingen af diabetes mellitus, søgtes behandlingssituationen eftergjort ved daglig – i en fire døgns periode – at injicere terapeutiske mængder af det mærkede insulin Også ved de sammensatte præparater fandtes betydelige variationer i absorptionshastigheden efter en enkelt injektion, ligesom der sås store variationer såvel inden for som mellem patienter

Det var ikke muligt at drage sikre slutninger om mekanismen ved absorptionen af de undersøgte protraherede præparater Det formodes, at der foregik en opløsning af insulinet in situ, hvorefter absorptionen fandt sted direkte fra væv til blod, men noget bevis herfor er ikke ført

Det må konkluderes, at undersøgelsen er for tidskrævende til, at den kan finde praktisk anvendelse til fastlæggelse af insulindosering hos den enkelte patient Det må på den anden side fremhæves, at den direkte undersøgelse af et præparats absorptionsegenskaber kan give oplysninger, som man ikke kan opnå på nogen anden måde

Kapitel 5

Den i et givet tidsrum fra et subcutant depot af opløst insulin absorberede insulinmængde viste en signifikant sammenhæng med de i samme tidsrum påviste ændringer i blodsukkerkoncentrationen Korrelationskoefficienten var imidlertid ikke stor Dette tages som udtryk for andre faktorers regulerende indflydelse på den faktiske blodsukkerkoncentration

Ved undersøgelsen af absorptionen af terapeutiske doser protraheret insulin givet til en mindre gruppe labile diabetikere fandtes tilsvarende, at den i

et givet tidsrum tilførte insulinmængde kun var bestemmende for en mindre del af den i samme tidsrum fundne variation i blodsukkerkoncentrationen

En undersøgelse af et givet insulinpræparats absorptionsforhold synes således ikke at byde på genveje til vurderingen af dette præparats egnethed i terapien af diabetes mellitus

References

Abel, J J *Proc nat Acad Sci (U S A)* 1926, *12* 132 (50)

Allen, R A, D B Smith & J E Hiscott *Radioisotope data* Oxford 1961, p 90–91 (12)

Altura, B M *Bibl anat (Basel)* 1966, *9* 12–16 (33)

Altura, B M & B W Zweifach *Bibl anat (Basel)* 1966, *9* 22–28 (33)

Aubertin, E, L Servantie & C Chassagnettes *C R Soc Biol (Paris)* 1939, *130* 484 (50)

Balodimos, M C, R H Williams *Amer J Med Sci* 1962, *243* 49 (12, 37, 38, 48)

Beecher, H K & A Krogh *Nature* 1936, *137* 458 (23)

Bermont, A *Complications dermatologiques de l insulinotherapie* Disputats Nancy 1967, p 54 & 57 (45)

Binder, C, Aa V Nielsen & K Jorgensen *Scand J clin Lab Invest* 1967 *19* 156 (12, 23, 37, 38, 48, 67)

Bischoff, F & L M Jemtegaard *Amer J Physiol* 1937, *119* 149 (50)

Blatherwick, N R, M E Ewing & P J Bradshaw *Amer J Physiol* 1938, *121* 44 (50)

Conn, H L *J appl Physiol* 1961, *16* 1065 (27)

Dobson, H L, L Robbins, Ph C Johnson, J Wdalel, D D Odem G Cornwall & Mary Davis *Metabolism* 1967, *16* 723 (12)

Efroymson, M A Multiple regressionanalysis Ralston, A & H S Wilf *Mathematical methods for digital computers* N Y Wiley 1960 p 191–204 (42)

Hagedorn, H C & B Norman Jensen *Biochem Z* 1923, *137* 92 (57)

Hagedorn, H C, B Norman Jensen, N B Krarup & I Wodstrup *J Amer med Ass* 1936, *106* 177 (50)

Hallas-Møller K, M Jersild K Petersen & J Schlichtkrull Dan med Bull 1954, *1* 132 (51)

Heding, Lise Sources of error in radioimmunoassay Donato, L G Milhaud & J Sirchis (Eds) *Labelled proteins in tracer studies* Brussels EURATOM 1966, p 249 (17, 26)

Hewitt, H B *Brit J exp Path* 1954 *35* 35 (21)

Himwich, H E, G S Goldman & M Y Krosnick *Amer J Physiol* 1932, *102* 365 (33)

Holzman, G B, H N Wagner, M Iio, D Rabinowitz & K L Zierler *Circulation* 1964, *30* 27 (35)

Hyman, C & Rita L Paldino *Bibl anat (Basel)* 1966, *9* 38 (33)

Ismail A A, M S EyRich, A A Hay, G Kamel, M Talaat & K I Fayek *Acta physiol Acad Sci hung* 1967, *31* 321 (35)

Joiner, C L Lancet 1959a i 964–67 (11, 23, 37 38, 48)

Joiner, C L & J D Pearson *Brit J Radiol* 1959b *32* 657 (12 23, 37 48)

Jorgensen, K & C Binder ^{125}I insulin as a tracer of insulin in different chemical processes Donato, L G Milhaud & J Sirchis (Eds) *Labelled proteins in tracer studies* Brussels, EURATOM 1966, p 329 (12)

Krarup, N B *Clinical investigations into the action of protamine insulinate* Disputats Kbh 1935 (50)

Krayenbühl, Ch. & T Rosenberg *Rep Steno Hosp* 1946, *1* 60 (51)
Lang, E.H & L.Reiner *Science* 1941, *93* 401 (12)
Lassen, N A., I F Lindbjerg & O Munck *Lancet* 1964, *i* 686. (27)
Lindbjerg, I F *Scand J clin. Lab Invest.* 1967, 19 120 (47)
Litton, A., C.W Walter & H J Berman *Bibl anat. (Basel)* 1966, *9* 50 (33)
Marcker, K. *Acta chem scand* 1960a *14* 194 (34)
Marcker, K. *Acta chem. scand* 1960b, *14* 2071 (34)
Moore, E.W, M.L.Mitchell & T C.Chalmers *J clin. Invest* 1959, *38* 1222 (12, 23, 37, 38, 48)
Munck, O, Anne Marie Andersen & C.Binder *Scand J clin Lab Invest* 1967, *suppl 99* 39 (35, 47)
Munck, O I F Lindbjerg C Binder N A Lassen & J Trap-Jensen *Diabetes* 1966, *15* 323 (27)
Nielsen, Aa.V & C.Binder *Acta med scand* 1967, *181* 521 27 (42)
Nora, J J, D W Smith & J R Cameron *J Pediat* 1964, *64* 547 (12, 37, 38 48, 66)
Orozlan, S I & Gertrude D Maengwyn Davies *Biochem biophys Acta (Amst)* 1962, *63* 117 (33)
Pfeiffer, E.F Recognized diabetogenic hormones and diabetes in man in Leibel, B S & G A.Wrenshall (Eds.) *On the nature and treatment of diabetes* 1965 Excerpta Med Found p 368. (74)
Reiner, L., E.H Lang E.W Irvine W Peacock & R.D Evans *J Pharmacol exp Ther* 1943 *78* 352 (12)
Rasio, E.A. C.L.Hampers, J S Soeldner & G F Cahill *J clin. Invest* 1967, *46* 903 (36)
Renkin, E.M *Amer J Physiol* 1955, *183* 125 (47)
Root, H F J W Irvine, R D Evans, L.Reiner & T M Carpenter *J Amer med Ass* 1944 *1* 84 (11, 38, 48)
Samols, E. Immunochemical aspects of insulin in Leibel B S. & G A.Wrenshall (Eds) *On the nature and treatment of diabetes* 1965 Excerpta Med Found. p. 227 (74)
Schlichtkrull, J *Insulin crystals* Disputats. Kbh. 1958, p. 61–66. (46, 51)
Schlichtkrull, J, O Munck & M.Jersild *Acta med scand* 1965, *177* 103 (51)
Schlichtkrull, J Insulin preparations with prolonged effect. Dorzbach, E (Ed.) *Handbuch der experimentellen Pharmakologie* Bd ? Insulin., (in press) Springer Verlag (51, 64, 66)
Schou, J *Subcutan absorption af lægemidler* Disputats. Kbh 1959 (32)
Schou, J *Pharmacol. Rev* 1961 *13* 441–64 (32)
Schriftman H & A.A.Kondritzer *Amer J Physiol* 1957, *191* 591 (33)
Scott, D A. *Biochem. J* 1934 *28* 1592 (50)
Scott, D A. & A M Fisher *J Pharmacol exp Ther* 1935 *55* 206 (50)
Scott, D A. & A.M Fisher *J Pharmacol. exp Ther* 1936, *58* 78 (50)
Seltzer H.S. Exhaustion of insulinogenic reserve in maturity-onset diabetes during prolonged and continuous hyperglycemic stress in Demole, M. (Ed.) *4e congrès de la Federation internationale du Diabète 1961* Editions médecine et hygiène. Genève p 650–53 (74)
Sund, R.B. & J Schou *Acta pharmacol (Kbh)* 1964a, *21* 313 (33)
Sund, R.B. & J Schou *Acta pharmacol (Kbh.)* 1964b, *21* 339 (33)
Trap-Jensen J J S Alpert, G del Rio & N A.Lassen *Acta med. scand* 1967, *suppl 476* 135 (49)
Warner, G F E.L.Dobson N Pale, M E.Johnston & Caroline R.Finney *Circulation* 1953, *8* 732 (33)
Williams, R H & F C.Wood Recent investigations and questions concerning diabetes in

References

Abel, J J *Proc nat Acad Sci (U S A)* 1926, *12* 132 (50)

Allen, R A , D B Smith & J.E Hiscott *Radioisotope data* Oxford 1961, p 90–91 (12)

Altura, B M *Bibl anat (Basel)* 1966, *9* 12–16 (33)

Altura, B M & B W Zweifach *Bibl anat (Basel)* 1966, *9* 22–28 (33)

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Balodimos, M C , R H Williams *Amer J Med Sci* 1962, *243* 49 (12, 37, 38, 48)

Beecher, H K & A Krogh *Nature* 1936, *137* 458 (23)

Bermont, A *Complications dermatologiques de l insulinotherapie* Disputats Nancy 1967, p 54 & 57 (45)

Binder, C , Aa V Nielsen & K Jorgensen *Scand J clin Lab Invest* 1967, *19* 156 (12, 23, 37, 38, 48, 67)

Bischoff, F & L M Jemtegaard *Amer J Physiol* 1937, *119* 149 (50)

Blatherwick, N R , M E Ewing & P J Bradshaw *Amer J Physiol* 1938, *121* 44 (50)

Conn, H L *J appl Physiol* 1961, *16* 1065 (27)

Dobson, H L , L Robbins, Ph C Johnson, J Wdalel, D D Odem G Cornwall & Mary Davis *Metabolism* 1967, *16* 723 (12)

Efroymson, M A Multiple regressionanalysis Ralston, A & H S Wilf *Mathematical methods for digital computers* N Y Wiley 1960, p 191–204 (42)

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Hagedorn, H C , B Norman Jensen, N B Krarup & I Wodstrup *J Amer med Ass* 1936, *106* 177 (50)

Hallas-Møller K , M Jersild K Petersen & J Schlichtkrull Dan med Bull 1954, *1* 132 (51)

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Hewitt, H B *Brit J exp Path* 1954, *35* 35 (21)

Himwich, H E , G S Goldman & M Y Krosnick *Amer J Physiol* 1932, *102* 365 (33)

Holzman, G B , H N Wagner, M Iio, D Rabinowitz & K L Zierler *Circulation* 1964, *30* 27 (35)

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Ismail A A , M S EyRich, A A Hay, G Kamel, M Talaat & K I Fayek *Acta physiol Acad Sci hung* 1967, *31* 321 (35)

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Joiner, C L & J D Pearson *Brit J Radiol* 1959b *32* 657 (12 23, 37 48)

Jorgensen, K & C Binder ¹²⁵I insulin as a tracer of insulin in different chemical processes Donato, L , G Milhaud & J Sirchis (Eds) *Labelled proteins in tracer studies* Brussels, EURATOM 1966, p 329 (12)

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Krayenbühl, Ch. & T Rosenberg *Rep Steno Hosp* 1946, *1* 60 (51)
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Lassen, N A., I F Lindbjerg & O Munck *Lancet* 1964, *1* 686 (27)
Lindbjerg, I F *Scand J clin Lab Invest* 1967, *19* 120 (47)
Litton, A., C W Walter & H J Berman *Bibl anat (Basel)* 1966, *9* 50 (33)
Marcker, K. *Acta chem. scand.* 1960a, *14* 194 (34)
Marcker, K *Acta chem. scand* 1960b, *14* 2071 (34)
Moore, E W, M L Mitchell & T C Chalmers *J clin Invest* 1959, *38* 1222 (12, 23, 37, 38, 48)
Munck, O, Anne Marie Andersen & C Binder *Scand J clin Lab Invest* 1967, *suppl* 99 39 (35, 47)
Munck, O, I F Lindbjerg C Binder, N A Lassen & J Trap-Jensen *Diabetes* 1966, *15* 323 (27)
Nielsen, Aa. V & C Binder *Acta med scand* 1967, *181* 521–27 (42)
Nora, J J, D W Smith & J R Cameron *J Pediat.* 1964, *64* 547 (12, 37, 38, 48, 66)
Oroszlan, S I & Gertrude D Maengwyn Davies *Biochem. biophys Acta (Amst)* 1962, *63* 117 (33)
Pfeiffer, E F Recognized diabetogenic hormones and diabetes in man in Leibel, B S & G A Wrenshall (Eds.) *On the nature and treatment of diabetes* 1965 Excerpta Med Found. p. 368. (74)
Reiner, L., E H Lang, E W Irvine, W Peacock & R D Evans *J Pharmacol exp Ther* 1943, *78* 352 (12)
Rasio, E A, C L Hampers, J S Soeldner & G F Cahill *J clin Invest* 1967, *46* 903 (36)
Renkin, E M *Amer J Physiol* 1955, *183* 125 (47)
Root, H F, J W Irvine, R D Evans L Reiner & T M Carpenter *J Amer med Ass* 1944, *1* 84 (11, 38, 48)
Samols, E. Immunochemical aspects of insulin in Leibel, B S & G A Wrenshall (Eds) *On the nature and treatment of diabetes* 1965 Excerpta Med Found. p 227 (74)
Schlichtkrull, J *Insulin crystals* Disputats. Kbh 1958, p. 61–66. (46, 51)
Schlichtkrull, J, O Munck & M Jersild *Acta med scand.* 1965, *177* 103 (51)
Schlichtkrull, J Insulin preparations with prolonged effect Dörzbach, E. (Ed.) *Handbuch der experimentellen Pharmakologie* Bd. ? Insulin., (in press) Springer Verlag (51, 64, 66)
Schou, J *Subcutan absorption af lægemidler* Disputats Kbh 1959 (32)
Schou, J *Pharmacol Rev* 1961, *13* 441–64 (32)
Schriftman H & A A Kondritzer *Amer J Physiol* 1957, *191* 591 (33)
Scott, D A. *Biochem. J* 1934, *28* 1592 (50)
Scott, D A. & A M Fisher *J Pharmacol exp Ther* 1935 *55* 206 (50)
Scott, D A. & A M Fisher *J Pharmacol exp Ther* 1936, *58*: 78. (50)
Seltzer, H S Exhaustion of insulinogenic reserve in maturity-onset diabetes during prolonged and continuous hyperglycemic stress in Demole, M (Ed.) *4e congrès de la Federation internationale du Diabète 1961* Editions medecine et hygiène Genève p 650–53 (74)
Sund, R B. & J Schou *Acta pharmacol (Kbh.)* 1964a, *21* 313 (33)
Sund, R B. & J Schou *Acta pharmacol (Kbh.)* 1964b, *21* 339 (33)
Trap-Jensen, J J S Alpert G del Rio & N A Lassen *Acta med scand* 1967, *suppl 476* 135 (49)
Warner, G F E L Dobson N Pale, M E Johnston & Caroline R Finney *Circulation* 1953, *8* 732 (33)
Williams, R H. & F C Wood Recent investigations and questions concerning diabetes in

man in Leibel, B S & G A Wrenshall (Eds) *On the nature and treatment of diabetes* 1965 Excerpta med Found p 748 (74)
Williams, R H The pancreas in Williams, R H *Textbook of endocrinology* Philadelphia 1968, p 658 (74)
Winter, C C *Radioisotope renography* Baltimore 1963, p 94–99 (12)

Subject index

man in Leibel, B S & G A Wrenshall (Eds) *On the nature and treatment of diabetes* 1965 Excerpta med Found p 748 (74)
Williams, R H The pancreas in Williams, R H *Textbook of endocrinology* Philadelphia 1968, p 658 (74)
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ACTA PHARMACOLOGICA
ET TOXICOLOGICA

VOLUMEN 27, SUPPLEMENTUM I, 1969

STUDIES ON THE METABOLISM OF METHYL MERCURY AND DIMETHYL MERCURY IN MICE

BY

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Studies on the metabolism of methyl mercury and dimethyl mercury in mice

From the Department of Food Hygiene,
the Department of Pharmacology and the Department
of Clinical Chemistry, Royal Veterinary College,
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TO MY WIFE

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Introduction

In 1852 Frankland discovered that mercury combines with methyl iodide in sunlight to form methyl mercury iodide (Frankland 1853) Following this discovery the advances in metal organic chemistry during the 19th century resulted in a variety of methods for the synthesis of different organo mercurials amongst which were methyl mercury (Buckton 1858 Schroeder 1879 Seidel, 1884) dimethyl mercury (e g Buckton 1858 1859 1863 Frankland & Duppa 1863) and phenyl mercury (e g Dreher & Otto 1870 Kekule & Franchimont 1872 Ladenburg 1874)

The pesticidal action of organo mercurials was soon discovered and attempts to treat seed with different organic mercury compounds in order to prevent attacks by micro organisms were first made during World War I (Riehm 1914 Spieckermann 1914) However the methods of seed dress ing had to be developed to diminish the handling hazards as well as the costs and the vast utilization of organo mercurials for this purpose com menced after the development of the liquid seed treatment process by Zade (Zade 1943) For reviews on the history of this evolution see Lind strom (1961) Ulfvarson (1961) and Torgeson (1967)

In Sweden mainly methyl mercury salts were utilized for seed disinfec tion resulting in pollution by large amounts of this compound in the environment Simultaneously an increasing pollution by different mercury compounds occurred in the rivers and lakes of Sweden as a result of the use of mercury in industry Thus paper pulp industry caused pollution by phenyl mercury compounds and electro-chemical industries caused pollution by inorganic and metallic mercury

The deleterious effects of the mercury contamination on the wild fauna were first pointed out by Borg (1958) who observed an increasing mortal ity in grain eating birds and their predators, due to poisoning by organo mercurials He concluded that the main source of mercury in these cases was dressed seed Borg et al (1965 a) and Borg et al (1965) brought atten tion to the alarming observation that higher contents of mercury were found in tissues of predatory animals than in tissues from their prey They suggested an enrichment process which was later convincingly documented (Borg et al 1969) In fish an extreme accumulation of mercury from the surrounding water as well as from the food has been shown to occur

Introduction

In 1852, Frankland discovered that mercury combines with methyl iodide in sunlight to form methyl mercury iodide (Frankland, 1853) Following this discovery the advances in metal organic chemistry during the 19th century resulted in a variety of methods for the synthesis of different organo mercurials, amongst which were methyl mercury (Buckton, 1858, Schroeder, 187[illegible] [illegible] 188[illegible]) dimethyl mercury (e g Buckton, *1858*, *1859*, 1863, Franklа[illegible]
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(Raeder & Snekvik, 1941, Boetius, 1960, Johnels et al, 1967, Hannerz, 1968) Fish in Swedish lakes and rivers have been shown to contain large amounts of mercury, the main part of which is present in the form of methyl mercury (Westoo, 1966, Noren & Westoo 1967) A correlation between pollution of the rivers by inorganic mercury and phenyl mercury and the amount of methyl mercury in tissues of fish living in the waters, has been demonstrated (Noren & Westoo, 1967) indicating the occurrence of a biosynthesis of methyl mercury The precise mechanism of the bio-methylation of mercury is not known but it has been shown that, under certain conditions, methyl mercury and dimethyl mercury can be obtained from inorganic mercury in the presence of micro organisms or extracts of micro organisms (Jensen & Jernelov, 1967, 1968, Wood et al, 1968)

The Swedish "mercury problem" has been subjected to comprehensive research work In this work we have only referred to literature of direct interest regarding the present investigation A more complete picture of the situation can be obtained from reports on symposia (Informations konferens rorande kvicksilver Stockholm 1965, The mercury problem Stockholm, 1966, Nordiskt symposium kring kvicksilverproblematiken Lidingo, 1968), and also from popular literature (Landell, 1968)

It is evident that the outlined development of an increasing contamination of the environment with mercury compounds involves considerable hazards to animals and man Especially distasterous is the transformation in nature of inorganic mercury and maybe other mercury compounds to methyl mercury, since alkyl mercury compounds are chemically stable even in the soil (Kimura & Miller, 1964) and are slowly excreted in different species (Friberg 1959, Swensson et al 1959 a, Swensson & Ulfvar son 1968 a 1968 b) This results in conditions for accumulation in the food chains

It is obvious that in order to master the mercury problem a detailed knowledge is required of the biological properties of the compounds in volved particularly the methylated mercurials For dimethyl mercury it is not possible to discuss relevantly its potential importance until the biological properties of the compound are known

The aim of the present investigation was twofold—

—to elucidate the basic metabolic properties of non toxic doses of dimethyl mercury,

—to contribute to the knowledge of the metabolism of methyl mercury by studying certain parameters in a laboratory animal

The parameters to be studied were the following

For methyl mercury

1 To determine the whole body retention and the excretion routes of mercury

2. To determine the influence of the total dose of stable methyl mercury on the excretion rate and on the distribution of mercury
3. To determine the influence of sex and physical activity on the excretion rate.
4. To determine the chemical form in which mercury occurs in tissues and excreta with an attempt to identify potential metabolites

For dimethyl mercury

1. To determine the retention the distribution and the excretion routes of mercury after intravenous injection and inhalation of dimethyl mercury
2. To investigate the chemical form in which mercury occurs in tissues and excreta and to identify potential metabolites.

Abbreviations used in this book for some organic mercury compounds and their formulae

Trivial name	Abbreviation	Formula
Diphenyl mercury	$\varphi Hg\varphi$	⌬ *Hg*-⌬
Dimethyl mercury	MeHgMe	CH_3HgCH_3
Ethyl mercury-	EtHg-	$CH_3CH_2Hg^{\circ}$
Phenyl mercury-	φHg-	⌬ Hg°
Methoxyethyl mercury-	MeOEtHg-	$CH_3OCH_2CH_2Hg^{\oplus}$
Methyl mercury	*MeHg-*	$CH_3Hg^{\oplus}$

E g methyl mercury hydroxide is referred to as MeHgOH and phenyl mercury cyanide as φHgCN

Comment To distinguish the very volatile dimethyl mercury from the remaining compounds, the latter are referred to in this work as "non-volatile mercury compounds" in spite of the relatively high volatility of some of the substances

Chapter I Labelled mercury compounds Synthesis and methods of purification

Experimental metabolic studies using radioisotopes necessitates the use of labelled compounds of the highest possible radiochemical purity This is the case both for metabolic studies and for elaboration of methods for extraction and chemical analysis The methods of thin layer chromatography described in Chapter II showed that the labelled mercury compounds, commercially available were often heavily contaminated with radiochemical impurities. Therefore methods for purifying the different compounds had to be developed Two of the substances dimethyl mercury and diphenyl mercury not commercially available had to be synthetised labelled and purified in the laboratory

The degrees of radiochemical purity specified were for MeHgOH and MeHgMe determined by scraping the plates and counting the radioactivity as described in Chapter II

For other substances to be used only as reference substances in chemical separations thin layer chromatographic separations were made in order to detect possible radiochemical impurities visible as spots on autoradiogram

Mercury compounds and isotopes

^{203}Hg labelled MeHgOH and ^{203}Hg labelled $Hg(NO_3)_2$ were obtained from AB Atomenergi Studsvik Sweden The specific activity varied between 0 36 and 1 60 mCi/mg mercury and 6 2 and 12 4 mCi/mg mercury respectively ^{203}Hg labelled φHgAc and $\varphi HgNO_3$ were obtained from The Radiochemical Centre Amersham England and from AB Atomenergi Studsvik Sweden respectively The specific activity varied for both compounds between 0 5 and 1.5 mCi/mg mercury

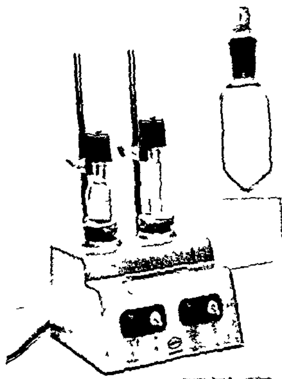
^{14}C labelled MeHgOH was synthetised by fil. kand Olof Borga at The Institution of Analytical Chemistry Stockholm University Stockholm Sweden The specific activity was 150 μCi/mg of mercury Unlabelled mercury compounds were kindly placed at our disposal by AB Casco Stockholm Sweden

Abbreviations used in this book for some organic mercury compounds and their formulae

Trivial name	Abbreviation	Formula
Diphenyl mercury	$\varphi Hg \varphi$	⌬-Hg-⌬
Dimethyl mercury	MeHgMe	CH_3HgCH_3
Ethyl mercury-	EtHg-	$CH_3CH_2Hg^{\oplus}$
Phenyl mercury-	φHg-	⌬-$Hg^{\oplus}$
Methoxyethyl mercury-	MeOEtHg-	$CH_3OCH_2CH_2Hg^{\bigcirc}$
Methyl mercury	MeHg-	$CH_3Hg^{\oplus}$

E g methyl mercury hydroxide is referred to as MeHgOH and phenyl mercury cyanide as φHgCN

Comment To distinguish the very volatile dimethyl mercury from the remaining compounds, the latter are referred to in this work as 'non-volatile mercury compounds" in spite of the relatively high volatility of some of the substances



ig 1 Test tube mixer used for shaking solutions in extraction procedures Inset 35 ml flask used for the extraction work

dure has not been found to be formerly described for dialkyl mercury compounds

MeHgI was prepared by shaking an aqueous solution of MeHgOH with benzene, after adding an excess of diluted hydroiodic acid The benzene layer was drawn off dried with anhydrous $MgSO_4$ and evaporated in a stream of nitrogen gas.

84 mg of solid MeHgI obtained in this manner was shaken for 60 minutes in 5 ml of pyridine together with an excess (50 mg wet weight) of freshly precipitated active copper powder (Blatt, 1948) washed several times in pyridine

Immediately after shaking the main part of the pyridine together with the formed dimethyl mercury, was distilled at 117° C and collected in a receiver immersed in hexane cooled to − 78° C

Precooling of the distillate was performed in a small, water-cooled glass condenser with small inner surfaces, specially built to reduce losses of substance at the surfaces (AB Werner Glas, Stockholm Sweden)

The labelling was carried out by shaking the distillate for 45 minutes at room temperature after adding 0 25 ml of a pyridine solution of $^{203}Hg(NO_3)_2$ This solution was obtained from an aqueous solution by evaporating the solvent in a stream of nitrogen gas and redissolving the substance in pyridine

Chemicals

Acetone p a , Merck
Ammonium hydroxide p a , ca 10 %, Merck
Benzene p a , Merck
Chloroform p a , Merck
Copper(II)sulphate cryst p a , Merck
n-Hexane purum, Merck
Hydrobromic acid, HBr, p a , density ca 1 50, Merck
Hydrochloric acid, HCl, p a , density ca 1 19, Merck
Magnesium(II)sulphate, $MgSO_4$, anhydrous, puriss , Merck
Nitric acid, HNO_3, p a , density ca 1 40, Merck
Nitrogen gas, AGA
Potassium cyanide, KCN, p a , Merck
Pyridine, p a , Mallinckrodt
Silver nitrate, p a , Merck
Sodium sulphate, Na_2SO_4 anhydrous, p a , Merck
Sodium hydroxide, NaOH, p a , Merck
Stannous(II)chloride, p a , Merck
All water used was distilled and deionized

Cleaning of glassware

All glass was first cleaned by ordinary laboratory procedures and then by shaking with 1 M ammonium hydroxide solution, with distilled water, and with ethanol

Equipment for extractions and phase separations

Extractions and phase separations were made in 10 ml test tubes fitted with ground-glass stoppers or in specially designed 35 ml Pyrex glass flasks (fig 1) with stoppers, heavy enough to be centrifuged with the stopper in place (AB Werner-Glas, Stockholm, Sweden)

The shakings of solutions were carried out in a Vortex Test Tube Mixer model K-500-2 (Scientific Industries, Inc , Springfield, Mass USA), redesigned to hold the test tube or flask and the stopper firmly during shaking (fig 1)

The centrifugings were carried out in a Kifa-Silverstolpe centrifuge model K-100 (AB Kifa, Stockholm, Sweden) for 5 minutes at a speed of 4000 rpm, corresponding to a field strength of 2200 to 2700 *g* This centrifuge is not influenced by an uneven load This is of value when working with delicate material since no time may be wasted upon taring before centrifuging

Synthesis, labelling and purification of ^{203}Hg-dimethyl mercury

The main principles for this semi-micro synthesis have been obtained from a macro method, described by Hein & Wagler (1925) The labelling proce-

chloroform The activity of the collected compound was ca 9 6 μCi, corresponding to a yield of > 19 % of the labelling compound The end product gave after treatment with diluted hydrochloric acid a compound, that behaved in phase partitions like phenyl mercury chloride, and could be converted to cyanide, which gave the same R_f value as phenyl mercury cyanide in thin layer chromatograms No radiochemical impurities could be detected by the thin layer chromatographic technique

Labelling and purification of ^{203}Hg-methoxyethyl mercury cyanide

Two ml benzene solution of 10 mg of methoxyethyl mercury chloride was shaken for 16 hours with 1 ml water solution of $^{203}Hg(NO_3)_2$ with a pH value of ca 1 The activity of the labelling compound was 200 μCi and the mercury content 0 02 mg The benzene layer was drawn off and shaken for 1 hour with an equal volume of 1 M KCN in water The benzene layer was collected and dried with anhydrous Na_2SO_4. The activity of the collected MeOEtHgCN in the benzene was 33 μCi corresponding to a yield of > 16 % of the labelling compound No radiochemical impurities could be detected by the thin layer chromatographic technique

Purification of ^{203}Hg-methyl mercury hydroxide and ^{14}C-methyl mercury hydroxide

The ^{203}Hg labelled MeHgOH commercially available, had, according to the manufacturer's specification in different batches a radiochemical purity varying between approximately 50 % and 95 % The purification procedure includes two steps the first one giving an end product with a radiochemical purity of > 97.5 % determined as described in Chapter II For most of the experiments this substance has been used and will be referred to as "MeHgOH" This method was also used for purifying the ^{14}C labelled MeHgOH

The second step gives a considerable loss of substance, but also an additional elimination of radiochemical impurities The radiochemical purity obtained after the second step has varied between 98 0 % and 98.5 % This substance has been used for some of the experiments with special requirements for a high degree of purity and will be referred to as "the twice purified MeHgOH"

Step 1 A water solution of methyl mercury hydroxide was acidified with hydrochloric acid to a final concentration of 0.5 M HCl The aqueous phase was extracted twice with half the volume of benzene by shaking for 10 minutes. The benzene phases were drawn off and put together and

The activity of the labelling substance was 5 0 mCi and the mercury amount 0 55 mg

When the shaking was finished the distillation procedure was repeated This time the distillation temperature was carefully held at about 96° C As soon as the temperature rose to 100° C, the procedure was interrupted The distillate was collected in 9 fractions together weighing 0 954 g The total activity of the distillate was determined by comparing aliquots with a ^{203}Hg standard by counting in a gamma spectrometer (Mark I, Nuclear Chicago, Des Plaines, Ill, USA) It was 2820 μCi, corresponding to a yield of >56 % of the activity in the labelling compound

The end product gave, when treated with diluted hydrochloric acid or with acetone solution of mercuric(II)chloride a compound that behaved in phase partitions like MeHgCl could be converted to cyanide, which gave the same R_f value as MeHgCN in thin layer chromatograms

The radiochemical purity, determined according to the method described in Chapter II, was >96 %

The specific activity was 150 μCi/mg Hg For this determination, the compound was converted to CH_3HgCl, with the aid of hydrochloric acid and the amount of CH_3HgCl determined by gas chromatography at Swedish Water and Air Pollution Research Laboratory, Stockholm

Synthesis, labelling and purification of ^{203}Hg-diphenyl mercury

The principles of the synthesis have been obtained from Maynard (1924) Two ml of 40 % (w/v) sodium hydroxide solution were slowly added to a solution of 0 5 g of stannous(II)chloride in 2 ml of water A suspension of 300 mg of phenyl mercury acetate in 0 5 ml of water was added and the mixture was shaken at room temperature for 60 minutes After centrifuging the supernatant was discarded The formed diphenyl mercury was extracted from the deposit with 2 ml of acetone by shaking for 10 minutes The acetone was collected after centrifuging and the diphenyl mercury precipitated by diluting the acetone drop by drop with water to a final volume of 20 ml The deposit was washed with water and centrifuged This procedure was repeated once After drying in a stream of nitrogen gas the crystalline deposit 65 mg was collected

For the labelling procedure 10 mg of the crystalline substance was dissolved in 2 ml of acetone and shaken for 12 hours at room temperature with 10 μl water solution of $^{203}Hg(NO_3)_2$ The activity of the labelling compound was 50 μCi and the mercury amount 0 05 mg

The diphenyl mercury was then precipitated by adding water as described above After centrifuging the deposit was dried in a stream of nitrogen gas Finally the diphenyl mercury was collected by dissolving in 2 ml of

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2 — (Oikos)

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For the labelling procedure 10 mg of the crystalline substance was dissolved in 2 ml of acetone and shaken for 12 hours at room temperature with 10 μl water solution of $^{203}Hg(NO_3)_2$ The activity of the labelling compound was 50 μCi and the mercury amount 0 05 mg

The diphenyl mercury was then precipitated by adding water as described above After centrifuging the deposit was dried in a stream of nitrogen gas Finally the diphenyl mercury was collected by dissolving in 2 ml of

chloroform The activity of the collected compound was ca 9 6 μCi, corresponding to a yield of > 19 % of the labelling compound The end product gave after treatment with diluted hydrochloric acid a compound, that behaved in phase partitions like phenyl mercury chloride, and could be converted to cyanide which gave the same R_f value as phenyl mercury cyanide in thin layer chromatograms No radiochemical impurities could be detected by the thin layer chromatographic technique.

Labelling and purification of ^{203}Hg-methoxyethyl mercury cyanide

Two ml benzene solution of 10 mg of methoxyethyl mercury chloride was shaken for 16 hours with 1 ml water solution of $^{203}Hg(NO_3)_2$ with a pH value of ca 1 The activity of the labelling compound was 200 μCi and the mercury content 0 02 mg. The benzene layer was drawn off and shaken for 1 hour with an equal volume of 1 M KCN in water The benzene layer was collected and dried with anhydrous Na_2SO_4. The activity of the collected MeOEtHgCN in the benzene was 33 μCi corresponding to a yield of > 16 % of the labelling compound No radiochemical impurities could be detected by the thin layer chromatographic technique

Purification of ^{203}Hg-methyl mercury hydroxide and ^{14}C-methyl mercury hydroxide

The ^{203}Hg labelled MeHgOH commercially available, had, according to the manufacturer's specification, in different batches a radiochemical purity varying between approximately 50 % and 95 % The purification procedure includes two steps, the first one giving an end product with a radiochemical purity of > 97 5 % determined as described in Chapter II For most of the experiments this substance has been used and will be referred to as "MeHgOH" This method was also used for purifying the ^{14}C labelled MeHgOH

The second step gives a considerable loss of substance but also an additional elimination of radiochemical impurities. The radiochemical purity obtained after the second step has varied between 98 0 % and 98 5 % This substance has been used for some of the experiments with special requirements for a high degree of purity and will be referred to as "the twice purified MeHgOH"

Step I A water solution of methyl mercury hydroxide was acidified with hydrochloric acid to a final concentration of 0.5 M HCl The aqueous phase was extracted twice with half the volume of benzene by shaking for 10 minutes The benzene phases were drawn off and put together and

shaken for 16 hours with an equal volume of an aqueous suspension of freshly precipitated silver oxide, thoroughly washed with water The aqueous phase was drawn off and centrifuged, and the clear supernatant, containing the purified MeHgOH, collected

Step 2 A water solution of the purified MeHgOH was acidified with hydrochloric acid to a final concentration of 0 5 M HCl and extracted twice with an equal volume of benzene by shaking for 10 minutes The benzene phases were put together and shaken for 30 minutes with half the volume of 1 M KCN in water After collecting the benzene layer the water phase was extracted by 10 minutes shaking with an equal volume of benzene The benzene phases, containing MeHgCN, were put together and shaken for 16 hours with half the volume of an aqueous suspension of freshly precipitated silver oxide, thoroughly washed with water The aqueous phase was drawn off and centrifuged and the clear supernatant containing the twice purified MeHgOH ', collected

Labelling and purification of ^{203}Hg-labelled ethyl mercury hydroxide

Ethyl mercury hydroxide was obtained from ethyl mercury chloride by dissolving in benzene and shaking with an aqueous suspension of silver oxide (as described above for MeHgOH) The water solution of EtHgOH was acidified with diluted nitric acid and the labelling was carried out by adding $^{203}Hg(NO_3)_2$ and shaking for 45 minutes Ethyl mercury was puri fied and obtained in the form of hydroxide according to a procedure analogous to the first step of the method described for MeHgOH No radiochemical impurities could be detected by the thin layer chromato graphic technique

Purification of ^{203}Hg-labelled phenyl mercury cyanide

φHgBr was prepared by shaking a benzene solution of φHgAc for 60 minutes with an equal volume of 0 5 M HBr The benzene phase was collected and evaporated in a stream of nitrogen gas The obtained solid φHgBr was repeatedly washed with 0 1 M HBr After drying in a stream of nitrogen gas and dissolving in benzene φHgCN was obtained by shaking the benzene phase for 16 hours with a water solution of 1 M KCN and collecting the benzene layer The benzene solution was dried with anhydrous $MgSO_4$

No radiochemical impurities could be detected in the fresh solution by the thin layer chromatographic technique After storing the solution for some time (months) an increasing amount of diphenyl mercury could be detected

Storage of the mercury compounds

In order to avoid chemical changes in the mercury compounds or losses through the high volatility of some of the substances certain precautions had to be taken in storing them

They were all, except the dimethyl mercury, kept in darkness at +4° C enclosed in glass vessels provided with tightly fitting screw caps.

The methyl mercury compounds were stored in the form of chloride in benzene solution, and, before use in animal experiments, converted to hydroxide by the method used in the first step of the purification procedure of MeHgOH

The dimethyl mercury was stored at +4° C in glass ampoules sealed by melting After opening an ampoule the contents were transferred to a glass vessel provided with a tightly fitting screw cap and stored at about −75° C

shaken for 16 hours with an equal volume of an aqueous suspension of freshly precipitated silver oxide, thoroughly washed with water. The aqueous phase was drawn off and centrifuged, and the clear supernatant, containing the purified MeHgOH, collected.

Step 2. A water solution of the purified MeHgOH was acidified with hydrochloric acid to a final concentration of 0.5 M HCl and extracted twice with an equal volume of benzene by shaking for 10 minutes. The benzene phases were put together and shaken for 30 minutes with half the volume of 1 M KCN in water. After collecting the benzene layer the water phase was extracted by 10 minutes shaking with an equal volume of benzene. The benzene phases, containing MeHgCN, were put together and shaken for 16 hours with half the volume of an aqueous suspension of freshly precipitated silver oxide, thoroughly washed with water. The aqueous phase was drawn off and centrifuged and the clear supernatant, containing the twice purified MeHgOH', collected.

Labelling and purification of ^{203}Hg-labelled ethyl mercury hydroxide

Ethyl mercury hydroxide was obtained from ethyl mercury chloride by dissolving in benzene and shaking with an aqueous suspension of silver oxide (as described above for MeHgOH). The water solution of EtHgOH was acidified with diluted nitric acid and the labelling was carried out by adding $^{203}Hg(NO_3)_2$ and shaking for 45 minutes. Ethyl mercury was purified and obtained in the form of hydroxide according to a procedure analogous to the first step of the method described for MeHgOH. No radiochemical impurities could be detected by the thin layer chromatographic technique.

Purification of ^{203}Hg-labelled phenyl mercury cyanide

φHgBr was prepared by shaking a benzene solution of φHgAc for 60 minutes with an equal volume of 0.5 M HBr. The benzene phase was collected and evaporated in a stream of nitrogen gas. The obtained solid φHgBr was repeatedly washed with 0.1 M HBr. After drying in a stream of nitrogen gas and dissolving in benzene, φHgCN was obtained by shaking the benzene phase for 16 hours with a water solution of 1 M KCN and collecting the benzene layer. The benzene solution was dried with anhydrous $MgSO_4$.

No radiochemical impurities could be detected in the fresh solution by the thin layer chromatographic technique. After storing the solution for some time (months) an increasing amount of diphenyl mercury could be detected.

counting The detection limit values and standard deviation of the radio activity measurements are listed in this chapter under "Determination of radiochemical purity"

Chemicals

Acetone p a Merck
Benzene p a Merck
Carbontetrachloride p a Merck
Diethyl ether anhydrous p a Mallinckrodt
2,5-diphenyl oxazole PPO Packard
Diphenylthiocarbazone (Dithizone) p a Merck
Petroleum ether (b p 40—60° C) p a Merck
Potassium cyanide KCN p a Merck
Silica Gel G (nach Stahl) Merck
Silica gel granulated with cobalt moisture indicator (desiccating agent)
Silver nitrate p a Merck
Toluene p a Merck

Cleaning of glassware

As described in Chapter I

Method A Thin-layer separation of organic mercury compounds of low volatility

A combined adsorption partition chromatography using water as stationary phase was used for separation of organic mercury compounds having a moderate volatility i e compounds that can be handled at room temperature during the separation process without noticeable losses of substance

Plates Glassplates (138×138 mm and 46×138 mm) were coated by means of a home made applicator with 0 4 mm layers of Silica Gel G The plates were allowed to dry overnight at room temperature and were then stored in a desiccator provided with desiccant (Silica gel) In order to obtain a suitable degree of moisture for the separation the plates were kept during the last 24 hours before use in a sealed glass vessel over water It was also possible to spray the dry plates with water immediately before the separation provided the spray was equally dispersed over the surface

Substances The compounds (except $\varphi Hg \varphi$) were applied as cyanides obtained from other derivatives (e g in extracts from biological material) by shaking a benzene phase over a water phase containing 1 M KCN The spots were applied 10 mm from the lower edge of the plates by means of

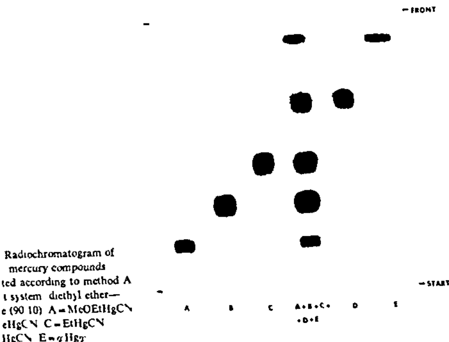
Chapter II. Chromatography and detection of radiomercury compounds

The necessity of reliable analytical methods for different mercury compounds in experimental studies concerning the metabolism of methyl and dimethyl mercury is obvious In searching for potential metabolites in extracts of organs and excreta the analytical procedure should include a method of separation, capable of complete separation of chemically and physically intimately related organic mercury compounds Since it is not possible to predict the chemical character of the metabolic products the method should include separation of alkyl and aryl compounds and must separate the compounds without destroying them or changing their chemical nature Furthermore, for investigations using radioactive compounds the separation method must allow a subsequent detection of the separated substances, utilising their radioactivity

Most of the methods for mercury analysis described in literature con cern the quantification of mercury independent of chemical state and do not involve any methods of separation between different mercury compounds (e g Stock, 1938 a, 1938 b, 1942, Maren, 1943, Vesterberg & Sjo holm, 1946, Sporek, 1956 Westermark & Sjostrand 1960, Ulfvarson, 1967, Toribara, 1968) In recent works, methods of separation have been described, using gas chromatography (Westoo, 1966, Sumino, 1968) thin layer chromatography (Westoo, 1966, Miettinen et al 1969 a), and electro phoresis (Westermark et al, 1968) None of these methods however, can fulfill the requirements stated

In order to make this metabolic study possible, methods of separation were developed to fulfill the demands stated Thin layer chromatography was chosen as a principle of separation with negligible influence on the chemical character of labile compounds Furthermore, this method offers simple and sensitive methods for the detection of radioactivity In order to determine the localization of radioactive spots the quickest method is scanning the chromatographic plate in a radiochromatogram scanner

A more exact method consists of exposing an X ray film, pressed against the plate which gives an autoradiogram of the chromatogram The adsorbent with the different spots on the plate can be scraped off, transferred to counting vials and the radioactivity of the spots determined by impulse



Radiochromatogram of
mercury compounds
ted according to method A
t system diethyl ether—
e (90 10) A = MeOEtHgCN
eHgCN C = EtHgCN
HgCN E = φHgφ

Plates Glassplates (138 × 138 mm and 46 × 138 mm) covered with a 0 4 mm layer of Silica Gel G The plates were dried for at least 18 hours at room temperature and then stored in a desiccator provided with desiccant before using

Substances The mercury compounds were applied in diethyl ether solu tion The dissociable compounds could be applied as cyanides or chlorides with the same result The technique of application was the same as described for method A

Development The development took place at − 75° C to − 78° C, using the upward flow technique in a preconditioned glass chamber The solvent system was a mixture of light petroleum ether and diethyl ether at a ratio of 95 5 (v/v) The distance between the origin and the front was 120 mm The time of development was about 25 minutes

Treatment of the plates after development After development the plates were transferred to room temperature and immediately sprayed with a 0 05 M $Hg(NO_3)_2$ solution in acetone + ether (80 20 v/v) and then with a 0 2 % (w/v) dithizone solution in acetone + ether (80 20 v/v) After drying the plates could be subjected to detection procedures

disposable pipettes (Microcaps® Drummond Scientific Co Broomall Pa USA) containing 1 5 or 10 μl depending on the concentration of the solution to be tested

Development Development took place at room temperature by vertical upward flow technique in a glass chamber presaturated with the solvent system The distance between the origin and the front was 120 mm The solvent system was a mixture of diethyl ether and toluene at a ratio of *90 10* (v/v) The development time was about 20 minutes

Treatment of the plates after development Immediately after development the plates were sprayed with 0 4 % (w/v) benzene solution of dithizone Hereby compounds (except φHgφ) present in amounts large enough to give visible colour reaction with dithizone appeared as bright yellow spots against a dark green background After drying the chromatoplates could be subjected to different detection procedures as described above

The R_f values obtained are dependent on the degree of moisture of the plates at development Therefore reference substances should be used and the following R_f values are approximate

Substance	R_f value
MeOEtHgCN	0 18
MeHgCN	0 34
EtHgCN	0 48
φHgCN	0 70
φHgφ	0 91

The mode of action of the mercury ion Hg^{2+} in this separation is of no interest since the inorganic mercury(II)cyanide is insoluble in benzene and inorganic mercury is consequently eliminated by the cyanide treatment of the substances before the chromatographic procedure

Fig 2 shows an autoradiogram of a chromatogram with the listed compounds separated according to method A

Method B Thin layer chromatographic separation of mercury compounds for the detection of dimethyl mercury

For the identification of the most volatile of the mercury compounds of interest in this study dimethyl mercury the thin layer chromatographic separation was carried out in a freezer in which the temperature varied between − 75° C and − 78° C

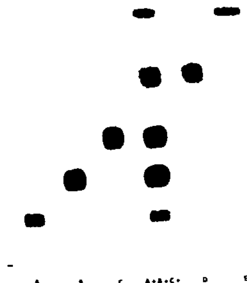


Fig 2 Radiochromatogram of organic mercury compounds separated according to method A Solvent system diethyl ether—toluene (90 10) A = MeOEtHgCN B = MeHgCN C = EtHgCN D = ϕHgCN E = ϕHgϕ

Plates Glassplates (138×138 mm and 46×138 mm) covered with a 0 4 mm layer of Silica Gel G The plates were dried for at least 18 hours at room temperature and then stored in a desiccator provided with desiccant before using

Substances The mercury compounds were applied in diethyl ether solution The dissociable compounds could be applied as cyanides or chlorides with the same result The technique of application was the same as described for method A.

Development The development took place at −75° C to −78° C, using the upward flow technique in a preconditioned glass chamber The solvent system was a mixture of light petroleum ether and diethyl ether at a ratio of 95 5 (v/v) The distance between the origin and the front was 120 mm The time of development was about 25 minutes

Treatment of the plates after development After development the plates were transferred to room temperature and immediately sprayed with a 0 05 M $Hg(NO_3)_2$-solution in acetone + ether (80 20 v/v) and then with a 0 2 % (w/v) dithizone solution in acetone + ether (80 20 v/v) After drying the plates could be subjected to detection procedures

disposable pipettes (Microcaps®, Drummond Scientific Co, Broomall, Pa, USA) containing 1, 5 or 10 μl, depending on the concentration of the solution to be tested

Development Development took place at room temperature by vertical upward flow technique in a glass chamber, presaturated with the solvent system The distance between the origin and the front was 120 *mm* The solvent system was a mixture of diethyl ether and toluene, at a ratio of 90 10 (v/v) The development time was about 20 minutes

Treatment of the plates after development Immediately after development the plates were sprayed with 0 4 % (w/v) benzene solution of dithizone Hereby compounds (except φHgφ) present in amounts large enough to give visible colour reaction with dithizone, appeared as bright yellow spots against a dark green background After drying, the chromatoplates could be subjected to different detection procedures as described above

The R_f-values obtained are dependent on the degree of moisture of the plates at development Therefore reference substances should be used and the following R_f-values are approximate

Substance*	R_f-value
MeOEtHgCN	0 18
MeHgCN	0 34
EtHgCN	0 48
φHgCN	0 70
φHgφ	0 91

The mode of action of the mercury ion Hg^{2+} in this separation is of no interest, since the inorganic mercury(II)cyanide is insoluble in benzene and inorganic mercury is consequently eliminated by the cyanide treatment of the substances before the chromatographic procedure

Fig 2 shows an autoradiogram of a chromatogram with the listed compounds separated according to method A

Method B Thin-layer chromatographic separation of mercury compounds for the detection of dimethyl mercury

For the identification of the most volatile of the mercury compounds of interest in this study, dimethyl mercury, the thin layer chromatographic separation was carried out in a freezer in which the temperature varied between − 75° C and − 78° C

— FRONT

— START

A B C A+B+C+D+E D E

Fig 2 Radiochromatogram of organic mercury compounds separated according to method A Solvent system diethyl ether—toluene (90 10) A = MeOEtHgCN B = MeHgCN C = EtHgCN D = φHgCN E = φHgφ

Plates Glassplates (138 × 138 mm and 46 × 138 mm) covered with a 0 4 mm layer of Silica Gel G The plates were dried for at least 18 hours at room temperature and then stored in a desiccator provided with desiccant before using

Substances The mercury compounds were applied in diethyl ether solution The dissociable compounds could be applied as cyanides or chlorides with the same result The technique of application was the same as described for method A

Development The development took place at − 75° C to − 78° C, using the upward flow technique in a preconditioned glass chamber The solvent system was a mixture of light petroleum ether and diethyl ether at a ratio of 95 5 (v/v) The distance between the origin and the front was 120 mm The time of development was about 25 minutes

Treatment of the plates after development After development the plates were transferred to room temperature and immediately sprayed with a 0 05 M $Hg(NO_3)_2$ solution in acetone + ether (80 20 v/v) and then with a 0 2 % (w/v) dithizone solution in acetone + ether (80 20 v/v) After drying the plates could be subjected to detection procedures

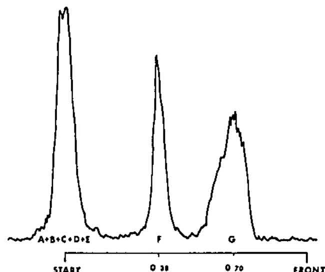


Fig 3 Diagram of radioactive thin layer chromatogram scanned in a radiochromatogram scanner showing the separation of dimethyl mercury from other mercury compounds according to method B Solvent system Petroleum ether —diethyl ether (95 5) Development temperature $-75°$ C A = $HgCl_2$ B = MeOEtHgCN C = MeHgCN D = EtHgCN E = φHgCN, F = φHgφ G = MeHgMe

Approximate R_f-values

Substance	R_f-value
HgX_2*	0
MeOEtHgX*	0
MeHgX*	0
EtHgX*	0
φHgX*	0
φHgφ	0 38
MeHgMe	0 70

* X = CN or Cl

Fig 3 represents a chromatogram made according to method B scanned in a radiochromatogram scanner

Method C Thin-layer chromatographic separation between organic and inorganic mercury dithizonates

This method was used for determination of the ratio between organic and inorganic mercury in biological material or in water solutions of organic mercury compounds (determination of radiochemical purity)

Plates Glassplates (138 × 138 mm and 46 × 138 mm) covered with a 0 25 mm layer of Silica Gel G The plates were dried and stored as the plates used in method B



Fig 4 Radiochromatogram showing separation of dithizonates of organic mercury from dithizonate of inorganic mercury according to method C Solvent system petroleum ether—acetone (90 10) A = φHg-dithizonate
B = MeHg dithizonate
C = Hg^{2+} dithizonate

Substances The substances were applied as dithizonates, obtained by shaking a water solution containing the compound or compounds with a 0 1 % (w/v) solution of dithizone in benzene or in carbontetrachloride Technically the application was performed as described for method A

Development Development at room temperature by upward flow technique in a presaturated glass chamber The distance between the origin and the front was 120 mm and the time of development about 20 minutes The solvent system was a mixture of light petroleum ether and acetone (90 10, v/v)

Treatment of the plates after development After drying at room temperature the plates could be subjected to determination procedures

Substance	R_f-value
Dithizonate of Hg^{2+}	0 22
Dithizonates of the organic mercury compounds used	0 36—0 40

Fig 4 represents a radiochromatogram according to method C of dithizonates of organic and inorganic mercury salts.

Method of determination of radiochemical purity

The radiochemical purity of methyl mercury compounds and of dimethyl mercury was tested by thin layer chromatography The different spots which were located by autoradiography of the chromatogram were scraped off with a razor blade and transferred to counting vials

For ^{203}Hg labelled compounds the radioactivity of the spots was measured in a gamma spectrometer

For ^{14}C labelled methyl mercury the counting vials contained 10 ml 0 5 % (w/v) PPO (2 5 diphenyl oxazole) in toluene and the measurements were carried out in a liquid scintillation counter (Mark II Nuclear Chicago Des Plaines Ill USA)

The radioactivity of the spots with the same R_f values as the standard substance was calculated as per cent of the total counts of the scraping from the origin to the front

For methyl mercury compounds the radiochemical purity stated is calculated with regard to the sum of the impurities found by method A and method C For dimethyl mercury method B has been applied for the detection

Detection limit and standard deviation in radiomercury determinations

All radiomercury determinations performed in this work except the whole body countings of mice were carried out in a Nuclear Chicago two channel gamma spectrometer (Mark I Nuclear Chicago Des Plaines Ill USA) at a counting efficiency of 22 7 % The background count was 21—25 cpm Detection limit can be defined as the standard deviation (near the detection limit) multiplied by the factor 1 64 (Lindstrom 1961) The chance that a blank will give an assay larger than the detection limit is then 1 in 20 in a single test

The total counting errors due to variation in background counts and depending on the number of counts in the sample can be calculated according to the formula

$$\text{Standard deviation of sample counting rate} = \pm \left[\frac{Cs}{T_s^2} + \frac{Cb}{T_p^2}\right]^{1/2}$$

(cit Comar 1955) where

C_s = total counts for the sample in time T_s
C_b – total counts for the background in time T_b

It is evident that the detection limit expressed in weight amount of mercury is dependent on the specific activity of the radioisotope used and that the standard deviation in analysis of a certain amount of mercury will

Table 1 Detection limits and standard deviation values when measuring ^{203}Hg labelled mercury compounds of different specific activities using gamma spectrometer

Specific activity of the compound	Detection limit		Standard deviation in analysis of 1 ng of mercury at counting time 10 min
	sample counting time 10 min	sample counting time 100 min	
100 μCi/mg Hg	0 08 ng	0 025 ng	± 0 064 ng
1 mCi/mg Hg	0 008 ng	0 0025 ng	± 0 015 ng
5 mCi/mg Hg	0 002 ng	0 0005 ng	± 0 0064 ng

increase with decreasing specific activity It is also evident that the counting time will have an influence on the accuracy of the measurements

Table 1 shows detection limits for 10 minutes and 100 minutes counting time using radioisotopes of the specific activities 100 μCi/mg 1 mCi/mg and 5 mCi/mg of mercury representative for the isotopes used in the experiments The table also shows the standard deviation in analysis of 1 mg of mercury of the three different specific activities when using the stated measurement equipment

Using ^{14}C labelled isotopes the detection limit will be somewhat lower and the standard deviation slightly less, since the counting efficiency will be higher between 50 and 60 % instead of about 23 % for ^{203}Hg

Specific activities of used compounds, sample counting time and standard deviation of the measurements will be stated in Chapter III or in description of the different experiments.

Method of determination of radiochemical purity

The radiochemical purity of methyl mercury compounds and of dimethyl mercury was tested by thin layer chromatography The different spots, which were located by autoradiography of the chromatogram, were scraped off with a razor blade and transferred to counting vials

For ^{203}Hg-labelled compounds the radioactivity of the spots was measured in a gamma-spectrometer

For ^{14}C labelled methyl mercury the counting vials contained 10 ml 0 5 % (w/v) PPO (2, 5 diphenyl oxazole) in toluene and the measurements were carried out in a liquid scintillation counter (Mark II, Nuclear Chicago, Des Plaines, Ill , USA)

The radioactivity of the spots with the same R_f-values as the standard substance was calculated as per cent of the total counts of the scraping from the origin to the front

For methyl mercury compounds, the radiochemical purity stated is calculated with regard to the sum of the impurities found by method A and method C For dimethyl mercury, method B has been applied for the detection

Detection limit and standard deviation in radiomercury determinations

All radiomercury determinations performed in this work, except the whole body countings of mice, were carried out in a Nuclear Chicago two channel gamma-spectrometer (Mark I Nuclear Chicago, Des Plaines Ill, USA) at a counting efficiency of 22 7 % The background count was 21—25 cpm Detection limit can be defined as the standard deviation (near the detection limit) multiplied by the factor 1 64 (Lindstrom, 1961) The chance that a blank will give an assay larger than the detection limit is then 1 in 20 in a single test

The total counting errors due to variation in background counts, and depending on the number of counts in the sample, can be calculated according to the formula

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C_s = total counts for the sample in time T_s
C_b = total counts for the background in time T_b

It is evident that the detection limit expressed in weight amount of mercury, is dependent on the specific activity of the radioisotope used and that the standard deviation in analysis of a certain amount of mercury will

Table 2 Specific activities, concentrations and levels of dosage of stable mercury in solutions of ^{203}Hg labelled MeHgOH given to mice

Specific activity	250 µl solution corresponds to		Dosage level when administering 250 µl of the solution to a 20 g mouse	Code for level of dosage
	activity	amount of mercury		
1670 µCi/mg Hg	1 µCi	0 6 µg	0 03 mg Hg per kilo body weight	A
167 µCi/mg Hg	1 µCi	6 0 µg	0 3 "	B
50 µCi/mg Hg	1 µCi	20 µg	1 0 "	C
10 µCi/mg Hg	1 µCi	100 µg	5 0 "	D

^{203}Hg labelled dimethyl mercury for injection and inhalation

For injection purposes, the stock solution was diluted with water at + 4° C so that 50 µl contained the activity desired to be given to each mouse.

For administration by inhalation, the stock solution was diluted with diethyl ether so that 25 µl contained the activity to be given to each mouse

Animals

Adult white mice of the american strain NMRI were used and mice weighing 20 g were selected for the investigations.

The mice were fed a complete pelleted mouse diet and water ad lib The pellets were supplied by AB Ewos, Sodertalje, Sweden and were according to the manufacturer composed of whey powder 5 %, expanded cereals 71 %, soybean oil meal 9 %, fish meal, defatted, 8 %, soybean oil 2 % yeast 2 %, lime stone 1 %, vitamin concentrate 1 %, trace elements 1 % *Average analysis dry matter* crude protein 20 %, crude fat 4 % ash 5 % (Ca 0 8 %, P 0 6 %), fiber 3 %, N free extracts 68 %, water content 8 %

Vitamins added (per 100 grams) were Vitamin A 360 IU, vitamin D 36 IU, vitamin E 4 2 IU, thiamine 150 micrograms, riboflavin 300 micrograms, pyridoxine 500 micrograms, pantothenic acid 500 micrograms, niacin 1 milligram choline chloride 0 1 gram

Trace elements added (per cent) were Fe 0 0016, Cu 0 00017, Co 0 000017, Mn 0 0016, I 0 00006, Zn 0 0126

The total mercury content of the feed was 20 ng/g and the mercury in the form of methyl mercury was 20 ng/g [1]

[1] The mercury analyses (in duplicate) were carried out at Swedish Water and Air Pollution Research Laboratory Stockholm

Chapter III. Materials and methods for the animal experiments

This chapter reviews the general materials used and methods employed. Details concerning single investigations will be described in connection with the account of the experiments.

Radioactive material

For specific activity and radiochemical purity of stock solutions, see Chapter I.

^{203}Hg labelled methyl mercury hydroxide for injection

For many of the experiments using methyl mercury compounds, certain levels of dosage with regard to stable mercury were desired. For that purpose, ^{203}Hg-labelled MeHgOH in water solution was diluted with unlabelled MeHgOH solution to give four different specific activities. The dilutions were regulated to give an activity of 1 μCi in the volume given to each mouse, 250 μl. Thus, four different levels of dosage of mercury were obtained when giving mice of the same weight equal doses of radioactivity. The levels of dosage of stable mercury, referred to as level A, B, C and D are listed in table 2 together with the most important data. For experiments with other dose requirements data will be given in connection with description of the experiments.

^{14}C-labelled methyl mercury hydroxide for injection

For injections of the ^{14}C labelled compound, the stock solution was diluted with water to give the activity of 1 μCi in 250 μl solution, the volume given each mouse. The corresponding mercury amount was 6 μg, corresponding to the dose 0.3 mg Hg per kilo body weight.

For injections of methyl mercury, labelled with ^{14}C and ^{203}Hg water solutions of ^{203}Hg-labelled MeHgOH and ^{14}C-labelled MeHgOH were diluted and mixed so that the volume given to each mouse, 250 μl, contained ca. 15 μCi of ^{14}C and ca. 5 μCi of ^{203}Hg. The total amount of mercury in 250 μl was 120 μg, corresponding to the dose 6 mg Hg per kilo body-weight.

Method of accomplishing physical activity in mice

Increased physical activity in the mice was accomplished by making them swim for three periods of 15 minutes each day in water temperated to 30° C The water containers used had the dimensions 33 cm×54 cm and the depth of water was 10 cm The exercise programme began one week before the start of the experiment and lasted until the measurement period was terminated

Method of sacrifice

The mice were sacrificed by immersion in gaseous carbon dioxide for thirty seconds

Sampling techniques

Blood sampling and exsanguination

The instruments used for blood sampling were for small volumes (e g for hematocrit determination) a capillary glass tube and for volumes over 100 μl (e g for collection of blood plasma or for exsanguination) a Pasteur pipette with the capillary end cut off and heated in a Bunsen flame to eliminate sharp edges

The sampling was performed by bleeding from the orbit under slight anaesthesia obtained by immersing the mouse for eight seconds in gaseous carbon dioxide The anaesthetised animal was grasped by the back of the neck and held securely to a flat surface The heparinised capillary glass tube was introduced into the lateral canthus and pushed through the orbital venous plexus producing a hemorrhage and the blood was collected with the glass instrument The bleeding was interrupted by removing the glass tube when the desired volume of blood was collected

Separation and sampling of feces and urine

Sampling for quantitative measurements on excreta For quantitative determinations of radioactivity in feces and urine the animals were kept two by two in metabolism cages with raised wire mesh floors (fig 5) The mice were fed from a tube system preventing crumbs from mixing with the excreta Feces fell through the wire mesh bottom against an oblique placed filter paper which transmitted the feces to a collecting Petri dish (diameter 20 cm) No detectable losses of radioactivity from the feces occurred in the intervals between collecting the excreta The filter paper (Munktell 333) was dusted with crystalline methylene blue (Merck) and

The mice were kept in cages provided with raised wire mesh floors to avoid recontamination from excreta

In experiments with dimethyl mercury the animals cages were kept in a fume cupboard In group experiments the animals were kept in groups of five in the cages The temperature in the animals cages was kept at 25° C to 28° C

Administration of substances to mice

Intravenous injection

Methyl mercury hydroxide in water solution was injected intravenously in a tail vein using a disposable tuberculine syringe attached to a syringe needle by means of a polyethylene catheter To determine the amount of solution injected the syringe with catheter was weighed before and after the injection

Dimethyl mercury was injected intravenously with the aid of a disposable 50 μl pipette (Microcaps® Drummond Scientific Co Broomall Pa USA) attached to a syringe with a polyethylene tubing system containing isotonic saline solution The dimethyl mercury was slowly injected with the aid of the saline solution

Oral administration

Oral administration of unlabelled mercury(II)nitrate in water solution was carried out by means of a disposable tuberculine syringe to which a blunt syringe needle was attached The concentration of the solution was adjusted to give the desired dose in a volume of 0 5 ml

Administration by inhalation

Dimethyl radiomercury was given to mice by inhalation using an apparatus according to Cohen and Hood (1969) and made by AB Werner Glas Stockholm Sweden The apparatus made from Pyrex® glass consisted of a container for the mouse connected to a glass bulb in which the ether solution of dimethyl mercury was deposited by means of a Hamilton syringe or a 25 μl disposable micro pipette The bulb was immersed in warm water (50° C) and the volatilization of the labelled compound was promoted by alternately pumping with two connected 5 ml syringes After 45 seconds the procedure was interrupted The mouse was then in a slight ether anaesthesia from which it recovered in a few minutes Usually between 50 % and 80 % of the deposited radioactive dimethyl mercury was transferred to the mouse by this process

Method of accomplishing physical activity in mice

Increased physical activity in the mice was accomplished by making them swim for three periods of 15 minutes each day in water temperated to 30° C The water containers used had the dimensions 33 cm×54 cm and the depth of water was 10 cm The exercise programme began one week before the start of the experiment and lasted until the measurement period was terminated

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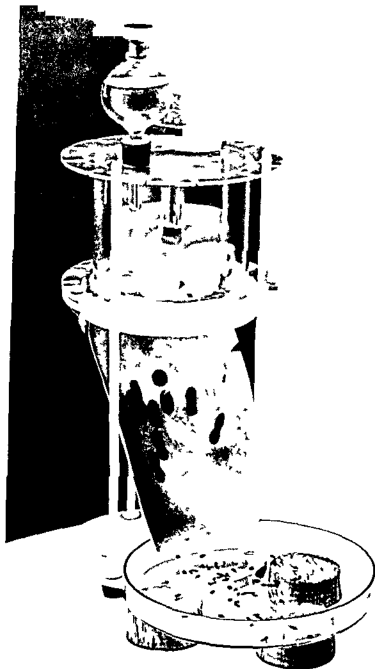


Fig 5 Metabolism cage used for separation and sampling of feces and urine from mice

sprayed with a 0 4 % (w/v) solution of dithizone in carbon tetrachloride When the urine falling through the mesh bottom hit the filter paper it was readily absorbed The particles of methylene blue dissolved in the urine thus giving bright blue spots, indicating the localisation of the absorbed urine The dithizone in the paper combined with methyl mercury compounds in the urine giving non volatile dithizonates and thus prevented losses of activity through volatilisation

The paper was renewed and the feces collected every 24th hour The spots containing urine were cut out and these spots and the collected feces transferred to tubes for radioactivity determinations

Sampling for qualitative determinations on excreta For qualitative chemical determinations on feces and urine the animals were kept one by one in the metabolism cages described above The collection of feces was performed in the previously described manner, the only difference being the filter paper, which in this case was not treated with any chemicals

The urine sampling was performed according to a method described by Pope (1967), using a latex rubber reservoir unit (Urine collection reservoir, Heyer Schulte Corp, Santa Barbara, Calif, USA) The unit had a flat bottom and dome shaped top with a volume of about 2 5 ml A side mounted latex tube (inner diameter ca 0 6 mm) was connected to a glass cannula of appropriate size

A reservoir was surgically implanted (under intravenous Mebumal® anaesthesia, 0 20—0 30 ml of a 0 5 % solution) in a subcutaneous position in the lumbar region of the mouse The tubing was positioned to run ventrally along the subcutaneous plane, then through the ventral peritoneum The cannula was inserted into the urinary bladder and secured by an encircling ligature After recovery of the animal, urine in the reservoir was obtained by inserting a sterile needle through the skin into the reservoir and withdrawing the fluid into a disposable syringe The placement of the dome seemed to be of no hindrance to the mice Mice with domes were able to move about without difficulty and made no attempt to remove domes by scratching or rubbing

The fluid was collected for three days from each animal before the commencement of the experiment, i e the injection of radioactive material In the experiments the urine was collected every 24th hour The volume of urine obtained daily from each mouse varied between 0 7 ml and 1 2 ml

Sampling of exhaled labelled compounds

Sampling for quantitative determinations on exhaled radiomercury after administration of dimethylradiomercury and methylradiomercury The essential parts of the experimental equipment will be seen from fig 6 Each mouse was placed in a cylindrical, 35 ml polyethylene container (AB Cerbo Plast Trollhattan Sweden), provided with holes for air intake, placed as shown in the figure This container was connected to three glass tubes, called A B and C (16 mm × 120 mm), a gas flow meter, a valve for regulation of the air stream, and a water suction pump All connections between the units were made airtight

For sampling after administration of ^{203}Hg labelled dimethyl mercury, the glass tubes called A and B (fig 6) were provided with 1 5 ml of a

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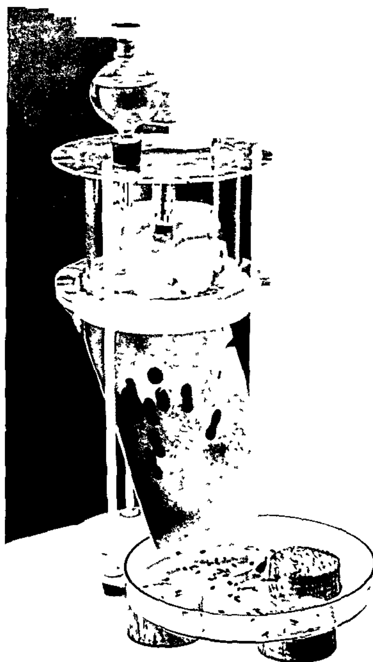


Fig 5 Metabolism cage used for separation and sampling of feces and urine from mice

sprayed with a 0.4 % (w/v) solution of dithizone in carbon tetrachloride When the urine falling through the mesh bottom, hit the filter paper, it was readily absorbed The particles of methylene blue dissolved in the urine, thus giving bright blue spots, indicating the localisation of the absorbed urine The dithizone in the paper combined with methyl mercury compounds in the urine giving non-volatile dithizonates and thus prevented losses of activity through volatilisation

(fig 6) consisted of one collecting tube cooled to −78° C by means of hexane and solid carbon dioxide The collection fluid was 1.5 ml of diethyl ether The gas flow rate was 0 4 liter × min⁻¹ During the sampling the moisture in the exhaled air was precipitated in the form of ice and exhaled mercury compounds dissolved to a large extent in the ether The ether was collected without raising the temperature dried with anhydrous sodium sulphate and transferred to glass vessels with tightly fitting screw caps and stored at −75° C for further treatment

Preparation of samples for radioactivity determination

Determination of gamma radiation

Radiomercury determinations in samples were carried out in a Nuclear Chicago gamma spectrometer Counting efficiency detection limit and standard deviation values are stated in Chapter II (p 26) Due to geometrical factors a decreasing counting efficiency was obtained if the height of the sample in a counting vial exceeded 12 mm. Therefore each sample was distributed to two or more counting tubes if they gave a sample height in one tube of more than 12 mm

For brain (including cerebellum and medulla oblongata) liver and kidneys the whole organs were measured after freeing them from extraneous fat and connective tissue

Muscle samples weighing 0 5—0 7 g were prepared from the posterior parts of the animals thighs

Blood samples of about 200 mg were transferred to counting tubes immediately after the blood collection

For measurements on blood plasma about 400 mg of blood was centrifuged for 2 minutes in a Spinco Microfuge Model 152 A (Beckman Instruments, Inc Palo Alto Calif USA) at a speed of 13 000 rpm (corresponding to a field strength of 6 000—11 000 g) and the obtained plasma submitted to radiomercury determination

Determination of gamma radioactivity in samples of urine and feces or in aliquots of extracts were performed after the collection without further treatment of the samples

Determination of beta radiation

Determination of beta radiation mainly from ^{14}C labelled compounds was performed in a liquid scintillation spectrometer (Mark II Nuclear Chicago des Plaines, Ill USA)

Preparation of samples for determination of $^{14}CO_2$ in exhaled air is described in connection with the description of CO_2 sampling (p 34)

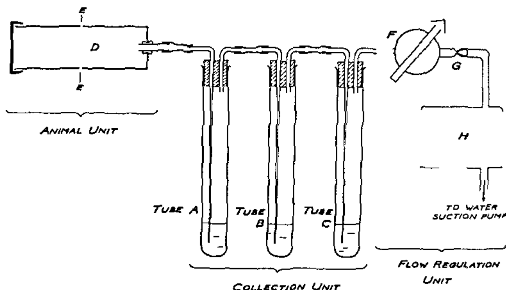


Fig 6 Equipment for sampling of exhaled labelled compounds (partly schematical) A, B and C = collection tubes D = container for mouse, provided with holes for air intake (E) F = gas flow meter, G = valve for regulation of gas flow rate, H = vacuum flask

0 05 M water solution of $Hg(NO_3)_2$ Glass tube C was provided with 1 5 ml of a 0 4 % (w/v) solution of dithizone in acetone.

For sampling after administration of ^{203}Hg-labelled methyl mercury the same solutions were used, though tube A and B contained the dithizone solution and tube C the solution of inorganic mercury

The gas stream was passed through the solutions by means of capillary glass tubes The gas flow rate was kept constant at 0 4 liter × min^{-1}

Sampling for quantitative determinations on exhaled $^{14}CO_2$ after administration of ^{14}C-labelled methyl mercury hydroxide Tube A in the collecting unit (fig 6) was replaced by a cold trap, cooled to −20° C in order to remove most of the moisture in the air The tubes B and C were replaced by collecting tubes, each with a volume of ca 30 ml, containing 10 ml of a phenetylamine solution, prepared according to Woeller (1961)

A mixture of 27 ml of redistilled phenetylamine (Mallinckrodt), 27 ml of absolute methanol, 500 mg 2, 5-diphenyl oxazole (PPO, Packard) and 10 mg 1, 4-bis [2-(5-phenyloxazolyl)]-benzene (POPOP, Packard) was diluted to 100 ml with toluene

The collection tubes A and B were cooled to 0° C in an ice bath The gas stream was dispersed in the phenetylamine solutions by means of sintered glass filters (porosity C)

Sampling for qualitative determinations on exhaled radiomercury after administration of ^{203}Hg-labelled dimethyl mercury The collecting unit

0 2 g of organic material (0 06 g of feces) was homogenised with water in a 5 ml glass homogeniser (AB Termo-Glas Goteborg, Sweden) and the homogenate was diluted with water to a volume of 2 ml 0 4 ml of concentrated HCl and 2 ml of benzene were added and the mixture was shaken for 5 minutes After centrifuging the benzene layer was drawn off and shaken for 5 minutes with 1 ml of 1 % solution of cysteine acetate saturated with sodium sulphate (prepared according to Westoo 1967) The clear part of the aqueous phase (ca 0 5 ml) was drawn off acidified with 0 5 ml 5 M HCl and extracted with 1 ml of benzene by shaking for 5 minutes. The benzene extract was dried with anhydrous sodium sulphate and submitted to thin layer chromatography

Method 2 *Extraction of volatile mercury compounds from animal tissues for subsequent chromatography*

This method involving a concentrating distillation step was used for qualitative determination and identification of dimethyl mercury in organic materials

The animal tissue (0 2—1 5 g) was homogenised at +4° C using a 30 ml glass homogeniser (AB Termo Glas Goteborg Sweden) and the homogenate was immediately transferred to the distillation equipment described in Chapter I (purification of dimethyl mercury) The water of the homogenate was partially (20—50 % depending on the volume of the homogenate) distilled to the receiver cooled to −78° C The obtained ice was melted and extracted with the smallest possible volume of diethyl ether at +4° C by shaking for 2 minutes The ether extract was dried with anhydrous sodium sulphate and submitted to chromatography (method B) The remaining part of the homogenate could be used for extraction according to Gage Westoo (p 36)

Method 3 *Procedure for determination of the extractable amount of radiomercury in tissues and excreta*

This method was designed for extraction of methyl mercury and was used only when extraction according to Gage Westoo and subsequent chromatography according to method A had revealed that the only organic radio mercury compound present was methyl mercury

The elaboration of the method was based on the following observations The phase separations are technically easier to perform after treatment of the tissue homogenate with strong alkali No significant decomposition of the methyl mercury ion occurs in 2 M NaOH at 100° C for 1 hour An increasing decomposition of the methyl mercury ion to inorganic mercury occurs with increasing concentration of HCl and when the temperature is raised The partition coefficient for methyl mercury between benzene

Determination of ^{14}C in chromatographic spots was performed as described for determination of radiochemical purity of ^{14}C labelled methyl mercury (p 26)

Hematocrit determination

For hematocrit determination heparinised whole blood was centrifuged for 5 minutes at 12 000 r p m in an Adams Autocrit centrifuge (Clay Adams Inc New York USA) Tests were always in duplicate and the mean was used The difference between duplicates was less than 1 per cent of the mean No correction was made for trapped plasma

SGOT—Determination

Serum Glutamate Oxalacetate Transaminase (SGOT) was determined according to Reitman and Frankel (1957) using commercially available reagents (AB Kabi Stockholm Sweden) The error of the method is about 4 2 % (Paulsson & Åberg 1965)

Histological examinations

The specimens for histological examination were fixed in 10 % formalin solution The fixed specimens were cut into 10—20 μ thick sections and stained with hematoxylin eosin scarlet red van Gieson s pichrofuchsin and Luxol fast blue

Extraction of mercury compounds from organic material

The essential parts of the equipment used in the methods in this work are described in Chapter I

Method 1 *Extraction of non volatile mercury compounds from organic material for subsequent chromatography*

This extraction method was used for subsequent chromatography for qualitative determination and identification of non volatile organic mercury compounds in organic materials

This extraction was performed according to a method described by Westoo (cysteine modification 1967) partly based on a method by Gage (1961) Because of the small samples used some alterations to the volumes of the liquid phases had to be made

0 2 g of organic material (0 06 g of feces) was homogenised with water in a 5 ml glass homogeniser (AB Termo Glas, Goteborg, Sweden) and the homogenate was diluted with water to a volume of 2 ml 0 4 ml of concentrated HCl and 2 ml of benzene were added and the mixture was shaken for 5 minutes After centrifuging the benzene layer was drawn off and shaken for 5 minutes with 1 ml of 1 % solution of cysteine acetate saturated with sodium sulphate (prepared according to Westoo, 1967) The clear part of the aqueous phase (ca 0 5 ml) was drawn off, acidified with 0.5 ml 5 M HCl and extracted with 1 ml of benzene by shaking for 5 minutes The benzene extract was dried with anhydrous sodium sulphate and submitted to thin layer chromatography

Method 2 *Extraction of volatile mercury compounds from animal tissues for subsequent chromatography*

This method, involving a concentrating distillation step, was used for qualitative determination and identification of dimethyl mercury in organic materials

The animal tissue (0 2—1 5 g) was homogenised at +4° C using a 30 ml glass homogeniser (AB Termo-Glas, Goteborg, Sweden) and the homogenate was immediately transferred to the distillation equipment, described in Chapter I (purification of dimethyl mercury) The water of the homogenate was partially (20—50 % depending on the volume of the homogenate) distilled to the receiver cooled to −78° C The obtained ice was melted and extracted with the smallest possible volume of diethyl ether at +4° C by shaking for 2 minutes The ether extract was dried with anhydrous sodium sulphate and submitted to chromatography (method B) The remaining part of the homogenate could be used for extraction according to Gage Westoo (p 36)

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The essential parts of the equipment used in the methods in this work are described in Chapter I

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This extraction method was used for subsequent chromatography for qualitative determination and identification of non volatile organic mercury compounds in organic materials

This extraction was performed according to a method described by Westoo (cysteine modification 1967) partly based on a method by Gage (1961) Because of the small samples used, some alterations to the volumes of the liquid phases had to be made

The mean and standard deviation for all the presented determinations after addition of methyl mercury in vitro and in vivo were

$$97.3 \pm 0.4\ \%$$

The value 97 % for extractable mercury according to this method was under certain conditions (further discussion in Chapter IX) regarded as an indication that practically all mercury present in the tissue was in the form of methyl mercury.

When using this method for feces or urine the extractability of methyl radiomercury from fresh homogenates was of the same order as for tissue homogenates but was found to diminish when a homogenate was stored for a couple of days. Thus the values of extractability obtained concerning excreta could not be regarded reliable as indications of the methyl mercury content. *However since the methyl mercury* content could not be lower than the obtained value of extractability the method was used for obtaining an estimation as to whether the main part of the mercury was *present in the methyl form* (Chapter VIII).

Whole body measurement of ^{203}Hg in mice

Measurement equipment

The determinations of whole body radioactivity (gamma activity) in mice were done with an apparatus the essential part of which consisted of two cylindrical thallium activated sodium iodide crystals with flat ends, facing each other at a distance of 45 mm. The dimensions of the crystals were 45×45 mm. They were electronically coupled (via FH 450 C) for summation of their individual counts to a scaler (Frieseke & Hopfner E 4/485). The crystals and detectors (FH 421 A) were encased in a lead shield (AB Kebo Stockholm Sweden) the thinnest part of which was 50 mm (fig. 7).

The specimen to be examined was placed in a 30 ml cylindrical polyethylene container (AB Cerbo Plast Trollhattan Sweden) provided with holes permitting respiration.

When measuring mice given radioactive dimethyl mercury certain precautions had *to be taken to eliminate exhaled* radiomercury. The polyethylene container for the mice was provided with air intake holes and a tube for evacuation as seen from the container in fig 6 (p 34).

The air was passed by means of a water suction pump through the container and the radioactive dimethyl mercury was absorbed by passing the air through two consecutive glass vessels each containing 200 ml of 0.05 M $Hg(NO_3)_2$ solution in water. The gas flow rate was 4—5 liters×min^{-1}. The specimen was placed equidistant from the two detectors at right angles to the axes of the crystals.

phase and aqueous phase has its maximum in the neighbourhood of pH 1 (K Östlund, unpublished observations and S Jensen, personal communication) and decreases considerably at lower pH values, thus rendering the extraction of methyl mercury from strongly acidic aqueous solutions more difficult The organic material was homogenised with water in a 30 ml glass homogeniser The concentration of the organic material in the homogenate was for tissues and urine 5 % and for feces 3 % An aliquot of the homogenate was taken out for radioactivity measurement 6 ml of the homogenate was transferred to a 30 ml glass flask with stopper and 2 ml of 8 M water solution of sodium hydroxide was added The flask was heated for 10 minutes in a briskly boiling water bath, and then cooled to room temperature 2 ml of 9 M hydrochloric acid was added and the mixture extracted with 5 ml of benzene by shaking for 3 minutes After centrifuging the benzene layer was collected and transferred to a weighed glass vessel *with stopper 0 8 ml of 9 M HCl* and 4 ml of benzene was added to the aqueous phase and the shaking and centrifuging procedures were repeated The benzene layer was drawn off and collected Finally, the aqueous phase was extracted twice with 4 ml of benzene The four benzene phases were put together, mixed and weighed The radioactivity was determined in aliquots of the total benzene phase and the aqueous phase and the radioactivity of the total phases were calculated The collected and weighed amount of benzene was regarded as the total amount of benzene used The aqueous phase was considered as being 10 8 ml

When ^{203}Hg-labelled MeHgOH was added to six different homogenates of liver the recovery in the benzene phase was 97 4 ± 0 6 % (mean ± s d) The recoveries from other tissues after addition of Me^{203}HgOH to homogenates were (mean of two determinations)

kidneys	97 4 %
brain	96 8 %
muscular tissue	97 3 %

When the determination was made in homogenates from organs of mice intravenously injected with methylradiomercury immediately before sacrificing the recovery values were (mean of two determinations)

liver	96 8 %
kidneys	97 0 %

No considerable differences seemed to occur between the different organs used and no considerable differences arose when the compound was injected into the animal, as compared to the values obtained after addition of the compound to the homogenate

was also used for the determination of radioactivity in parts of mice (skin with fur) In some of these samples the activity was low and a standard deviation of ±4 % had to be accepted

Treatment of whole-body measurement data

Calculation of retained amount of administered mercury

The amount of mercury retained in the mouse at the time of measurement (time T_1) was calculated as per cent of the administered dose (given at time T_0) according to the formula

$$\text{retained per cent of given dose} = \frac{A_{T_1} \; S_{T_0} \; 100}{S_{T_1} \; A_{T_0}}$$

where A_{T_1} = whole body counts for the mouse at time T_1
S_{T_0} = counts for the standard at time T_0
S_{T_1} = counts for the standard at time T_1
A_{T_0} = whole body counts for the mouse at time T_0
(all values in cpm and corrected for background)

Thus the obtained percentage value was corrected for radioactive decay as well as for background counts This percentage value is referred to as "% retained dose" and the $^{10}\log$ of the value as "log % retained dose"

Principles of excretion mechanisms regarded as mathematical functions Calculation of rate constants and biological half life

When "log % retained dose" is plotted against time (t) it gives a retention curve the principle of which is illustrated in fig 8 (unbroken line) The declination of the curve depends on the elimination of radiomercury from the animal by means of excreta Simultaneously to this excretion mercury is built in to the hairs of the fur (Chapter IV) The mercury, built in to hairs is removed from the body withdrawn from the circulation and may then be regarded as excreted Consequently the radioactivity* of the mouse measured by whole body counting consists of the sum of radio activity within the body (this activity called b) and the accumulated activity in the fur (called f)

Concurrently there is a diminishing of radioactivity of the fur depending on the replacement of hairs The velocity of this process may be considered negligible compared to the other excretion functions in the early stages of the course and will be neglected in the mathematical treatment of the reactions, though it will be mentioned in the discussion

* All values corrected for radioactive decay

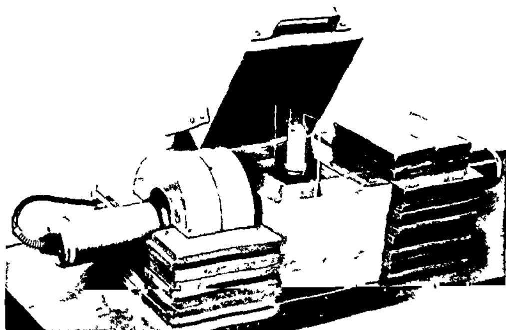


Fig 7 Detection part of measurement equipment for whole body determination in mice The holder for the container with mouse in place is elevated in order to show the placement of the specimen

The counting efficiency of this arrangement was about 12 % and the background count was about 800 cpm The sample counting time was one minute in most measurements It was extended to two or four minutes towards the end of the experimental periods in order to reduce the statistical errors of the determinations when the activity grew lower

Standards

Standards were prepared from water solutions of $^{203}Hg(NO_3)_2$, filled in glass tubes, sealed by melting The glass tubes were dimensioned to fit into the polyethylene containers used for the mice at whole-body counting The radioactivity of the standard for each experiment was adjusted to be of the same magnitude as the activity of the specimens

Statistical errors of the radioactivity measurements

The total counting errors calculated as described in Chapter II (p 26) were lowest in the early stages of the investigations when the whole body activity was high Standard deviation of sample counting rate was then about ± 0 25 % Towards the end of the experimental periods when the activity grew lower the standard deviation rose to about ± 2 %

In the experiment described in Chapter IV the measurement equipment

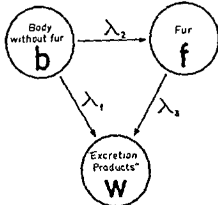


Fig 9 Schematical illustration of the compartments involved in the process of elimination of radiomercury after an i v injection of $Me^{203}HgOH$ in mice. Arrows indicate the directions of mercury transport λ_1 λ_2 and λ_3 are the rate constants of the processes b f and w are symbols for the radioactivity of the three compartments

Therefore in the following the approximation is made so that the retention curve for activity b is considered as being a straight line (dotted in fig 8)

The elimination of mercury from the body, regulated by the sum of the excretion mechanisms (including the transfer to the fur) is then kinetically a function of the first order

The rate constant of this total excretion function constituting the sum of λ_1 and λ_2 is called λ λ is the regression coefficient of the dotted line in fig 8 The biological half life of methyl mercury in the body without fur is then

$$T_{1/2} = \frac{\ln 2}{\lambda}$$

Since the sum of the excretion functions constitutes a function of the first order we may also assume that the components of this function are of the first order i e that λ_1 and λ_2 represent rate constants for reactions of the first order

The deviation of the retention curve (derived from the whole body measurement data) from the straight line corresponding to the mercury retention in the body without fur is dependent on the increasing amount of accumulated radiomercury in the fur The degree of deviation is dependent of the relation between λ_1 and λ_2

The accumulation of mercury in the fur will cease when the mercury amount b is negligible compared to f After this point of time the regression coefficient of the curve is of no interest in this investigation

In order to derive the value of $T_{1/2}$ from the whole body measurement data (b + f) the following operations were applied *

* This mathematical treatment and the data processing was carried out by fil kand Karl Hugo Eriksson Research Institute of National Defense

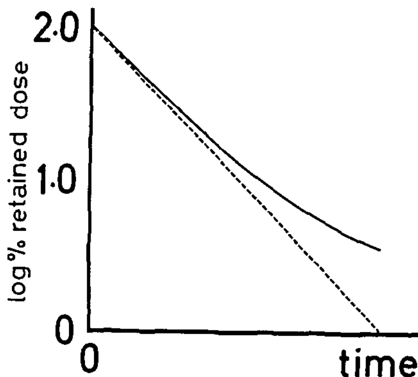


Fig 8 Principle of retention of radiomercury in mice after an i v injection of $Me^{203}HgOH$ Log % retained dose plotted against time Unbroken curve cor responds to whole body activity measurements broken line corresponds t the retention of mercury in the body without fur

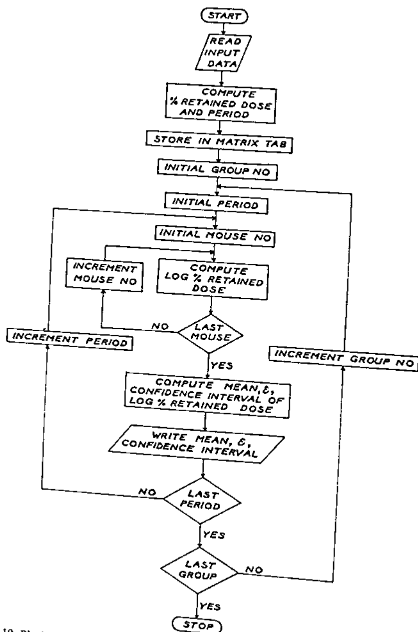
Fig 9 schematically shows the routes of elimination of mercury from the body The rate constants for the mechanisms are called λ_1, λ_2, and λ_3 λ_1 is the rate constant for the sum of the elimination mechanisms via excreta, i e feces, urine and potentially the exhaled air λ_2 is the rate constant for the building-in mechanism to the fur and λ_3 the constant for loss of mercury from the fur to 'excretion products' (activity w)

It will be seen from retention curves like fig 8 obtained from the experiments that the curve fairly well resembles a straight line in the initial part, i e when the amount of mercury in the fur is still negligible compared to the mercury in the body

This observation suggests that the retention curve may be a straight line if the measurement of radioactivity could comprise only the activity b and not as in the whole body counting include (b + f)

The experiment with whole-body counting and measurement of the activity in the integument and the fur of the same mouse (Chapter IV) adds validity to this assumption It is also in agreement with conclusions by Ulfvarson (1962) concerning methyl mercury and based on the fact that compounds which are not altered chemically in the organism and distribute freely between the organs may be supposed to be excreted with a rate proportional to the total body concentration in each moment

Thus it seems at any rate clear that the retention curve valid for mercury in the body itself (b) does not deviate from the straight line to any con siderable degree



ıg 10 Block diagram for data description Programme for calculation of data for raphic presentation of whole body measurement values

As a result of the preceding discussion and given definitions the following differential equation could be stated

$$\frac{db}{dt} = -(\lambda_1 + \lambda_2)\ b \qquad (t = \text{time}) \tag{1}$$

Thus $b = b_o\ e^{(\lambda_1+\lambda_2)t}$ (2)

(b_o = b at time zero e = base of natural logarithm system)

Consider the changes in activity w

If all activities are expressed in per cent of initial dose (= 100 %) the activity w is given by $w = 100 - (b + f)$ (3)

Thus in accordance with (1)

$$\frac{dw}{dt} = \lambda_1\ b \tag{4}$$

From (2) and (4) $w = \dfrac{\lambda_1\ b_o}{-(\lambda_1 + \lambda_2)}\ e^{(\lambda_1+\lambda_2)t} + C$ (5)

(C = arbitrary constant)

At time t = 0 w = 0 Thus $C = \dfrac{\lambda_1\ b_o}{\lambda_1 + \lambda_2}$ (6)

From (5) and (6) $w = \dfrac{\lambda_1\ b_o}{\lambda_1 + \lambda_2}\left[1 - e^{(\lambda_1+\lambda_2)t}\right]$ (7)

For further treatment the following abbreviations are used

$$\frac{\lambda_1\ b_o}{\lambda_1 + \lambda_2} = \alpha \tag{8}$$

$$\lambda_1 + \lambda_2 = k \tag{9}$$

Thus (7) $w = \alpha(1 - e^{kt}) = \alpha - \alpha\ e^{kt}$ (10)

In the following the measurement data from every single animal was treated separately (individually)

The whole body activity measurements of the animals concerned were performed at time zero and then every 24th or every 48th hour for a couple of weeks (or longer time)

In order to get a first approximation of the coefficients α and k to make possible a later iteration the principles of a method described by Williams (1964 a) were used

For α

The lowest measurement value (% retained dose) obtained at the end of the experimental period was set equal to $(100 - \alpha)$ since when $t \to \infty$ $100 - (b + f) \to \alpha$ [from (10)]

For k

From the first approximation of the α value the k value was roughly estimated using the measurement values from two different times of

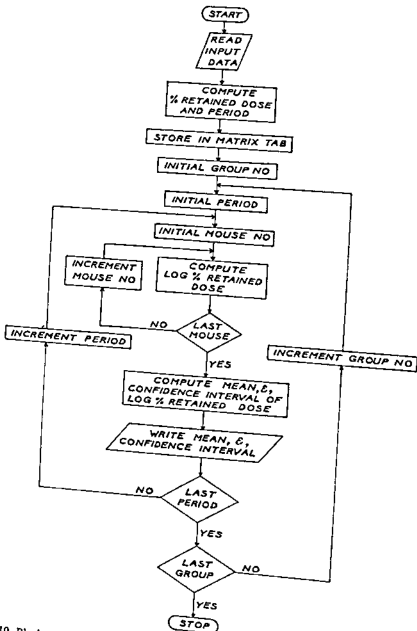


Fig 10 Block diagram for data description Programme for calculation of data for graphic presentation of whole-body measurement values

measurement in the initial course of the experiment (the second and fifth values from measuring times t_2 and t_5 respectively)

$$w_2 = \varkappa - \lambda e^{-\Lambda t_2} \tag{11}$$

$$w_5 = \varkappa - \lambda e^{-\Lambda t_5} \tag{12}$$

(indices for times of measurement)

$$\text{Thus } \Lambda = \frac{\ln\left(\frac{w_2 - \varkappa}{w_5 - \varkappa}\right)}{t_5 - t_2} \tag{13}$$

In order to more precisely estimate the coefficients λ and Λ, an iterative method described by Williams (1964 b) was applied

$$\text{Set } p = e^{-\Lambda t} \tag{14}$$

$$p' = -t\ e^{-\Lambda t} \tag{15}$$

$$\left(p' = \frac{dp}{d\Lambda}\right)$$

$$\text{Set } w = \varkappa + \varrho_1(p + \delta\Lambda p') = \varkappa + \varrho_1 p + \varrho_2 p' \tag{16}$$

(14) is a linear regression on p and p' For the calculation of the coefficients ϱ_1 and ϱ_2 the method of least squares was used

An adjusted value of Λ for next iteration was determined $\left(= \Lambda + \delta\Lambda,\ \text{where } \delta\Lambda = \frac{\varrho_2}{\varrho_1}\right)$

Then subsequent iterations were performed until the numeric value of $\delta\Lambda$ was less than 0 00005

In order to facilitate checking of the obtained functions, values of λ_1 and λ_2 were calculated by the equation system consisting of equations (8) and (9)

From the final Λ-value, the biological half-life was calculated $\left(T_{1/2} = \frac{\ln 2}{\Lambda}\right)$ and from the individual $T_{1/2}$ values for each mouse the mean and standard deviation for the different groups were calculated

Data processing of obtained whole body measurement values

Data processing was used to calculate values of whole body measurement for graphic presentation and to calculate biological half-life of radiomercury after administration of labelled methyl mercury hydroxide The programmes were written in the programming language FORTRAN IV and were run on the IBM 360/75 computer at Stockholm Data Center

The input data consisted of punched cards There was one card for every mouse and time of measurement Each card contained an identifica

tion number (i e group of mice No, mouse No and sequence No), whole-body activity (cpm) and elapsed time (after administration) expressed in days, hours and minutes

The output from the programme which processed data for graphic presentation of measurement values consisted of

for each group and period mean, standard error (ε) and 95 % confidence interval of log % retained dose

The output from the programme for computing $T_{1/2}$ consisted of

for each mouse γ, A, residual variance (RVAR), number of iterations (nit) λ_1 λ_2 and $T_{1/2}$

for each group of mice Mean of A and of $T_{1/2}$, variance of A and of $T_{1/2}$ standard deviation (s.d) of A and of $T_{1/2}$, and degrees of freedom (d f) of the variances

Block diagrams of the data programmes are presented in fig 10 and fig 11 The complete description of the programmes is available in a report by Eriksson (1969)

Whole-body autoradiography

Thirthy six mice, 15 adult female, 9 adult male and 12 pregnant female mice were used in the investigation The pregnant mice were given the labelled compound 2 days (11 animals) and 6 days (1 animal with longer time of survival) before expected delivery

Thirty one of the animals 13 female, 7 male and 11 pregnant mice were given the dimethyl mercury by intravenous injection To 5 animals, 2 female, 1 pregnant female and 2 male mice, the substance was administered by inhalation The doses varied between 19 and 52 μCi, corresponding to 0 13—0 35 mg of mercury The mice were sacrificed after 5 and 20 minutes and 1 4 and 24 hours and 4 and 16 days after the administration of labelled substance in accordance with the schedule shown in table 3

After anaesthetising by immersion in gaseous carbon dioxide the mice were sacrificed by immersion into a mixture of hexane and solid carbon dioxide (−78° C) The mice were mounted in a mixture of carboxymethyl cellulose and water, frozen to a solid block The autoradiographic technique by Ullberg (1954 1958) was used but had to be slightly modified as follows because of the high volatility of dimethyl mercury

The embedded mouse was sagittally divided in two equal halves, using an electric saw machine with a high speed, circular saw blade (diameter 20 cm thickness 2 5 mm) The plane surface of one half of the mouse was pressed against X ray film (Kodirex Kodak) The preparation, the exposure and all dark room work except developing, was carried out in a freezer at about −75° C The distribution patterns obtained in this manner may be regarded as representing the distribution of dimethyl mercury and potential metabolites

measurement in the initial course of the experiment (the second and fifth values from measuring times t_2 and t_5 respectively)

$$w_2 = \varkappa - \varkappa\ e^{-\Lambda t_2} \qquad (11)$$

$$w_5 = \varkappa - \varkappa\ e^{-\Lambda t_5} \qquad (12)$$

(indices for times of measurement)

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An adjusted value of Λ for next iteration was determined ($= \Lambda + \delta\Lambda$ where $\delta\Lambda = \frac{\varrho_2}{\varrho_1}$)

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Data processing was used to calculate values of whole body measurement for graphic presentation and to calculate biological half life of radio mercury after administration of labelled methyl mercury hydroxide The programmes were written in the programming language FORTRAN IV and were run on the IBM 360/75 computer at Stockholm Data Center

The input data consisted of punched cards There was one card for every mouse and time of measurement Each card contained an identifica

tion number (i e group of mice No mouse No and sequence No) whole body activity (cpm) and elapsed time (after administration) expressed in days hours and minutes

The output from the programme which processed data for graphic presentation of measurement values consisted of

for each group and period mean standard error (ϵ) and 95 % confidence interval of log % retained dose

The output from the programme for computing $T_{1/2}$ consisted of

for each mouse α 1 residual variance (RVAR) number of iterations (nit) λ_1 λ_2 and $T_{1/2}$

for each group of mice Mean of 1 and of $T_{1/2}$ variance of 1 and of $T_{1/2}$ standard deviation (s d) *of* 1 *and of* $T_{1/2}$ *and degrees of freedom* (d f) of the variances

Block diagrams of the data programmes are presented in fig 10 and fig 11 The complete description of the programmes is available in a report by Eriksson (1969)

Whole body autoradiography

Thirthy six mice 15 adult female 9 adult male and 12 pregnant female mice were used in the investigation The pregnant mice were given the labelled compound 2 days (11 animals) and 6 days (1 animal with longer time of survival) before expected delivery

Thirty one of the animals 13 female 7 male and 11 pregnant mice were given the dimethyl mercury by intravenous injection To 5 animals, 2 female 1 pregnant female and 2 male mice the substance was administered by inhalation The doses varied between 19 and 52 μCi corresponding to 0 13—0 35 mg of mercury The mice were sacrificed after 5 and 20 minutes and 1 4 and 24 hours and 4 and 16 days after the administration of labelled substance *in accordance with the schedule shown in table 3*

After anaesthetising by immersion in gaseous carbon dioxide the mice were sacrificed by immersion into a mixture of hexane and solid carbon dioxide (−78° C) The mice were mounted in a mixture of carboxymethyl cellulose and water frozen to a solid block The autoradiographic technique by Ullberg (1954 1958) was used but had to be slightly modified as follows because of the high volatility of dimethyl mercury

The embedded mouse was sagittally divided in two equal halves using an electric saw machine with a high speed circular saw blade (diameter 20 cm thickness 2 5 mm) The plane surface of one half of the mouse was pressed against X ray film (Kodirex Kodak) *The preparation the* exposure and all dark room work except developing was carried out in a freezer at about −75° C The distribution patterns obtained in this *manner may* be regarded as representing the distribution of dimethyl mercury and potential metabolites.

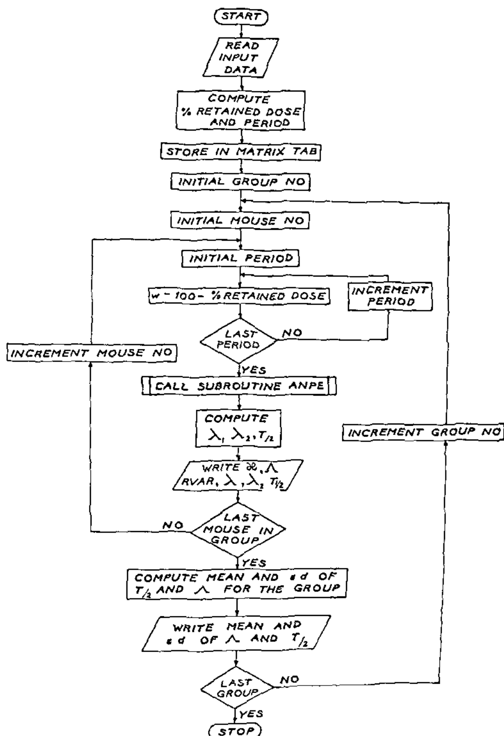


Fig 11 a

Fig 11 a b Block diagrams for data description Programme for calculation of biological half life ($T_{1/2}$) of radiomercury in the body without skin and fur (fig

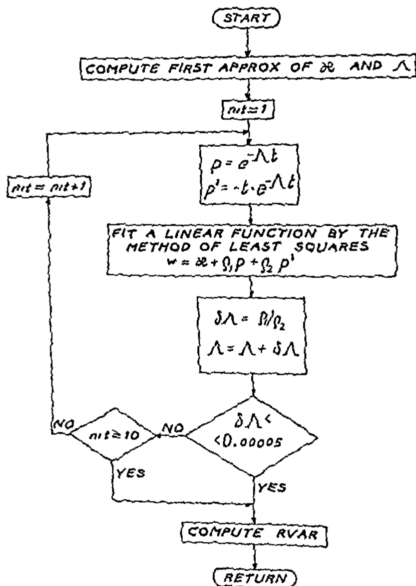


Fig 11 b

11 a) using subroutine ANPE (fig 11 b) for fitting a function $w = \varkappa(1 - e^{-\Lambda t})$
nit = number of iterations

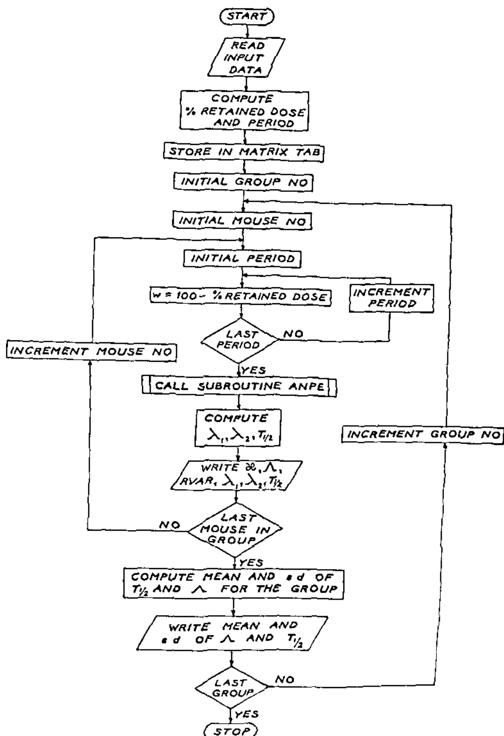


Fig 11 a

Fig 11 a, b Block diagrams for data description Programme for calculation of biological half-life ($T_{1/2}$) of radiomercury in the body without skin and fur (fig

x: single observation

n: number of observations

$\bar{x}$ mean of x, $\bar{x} = \frac{\Sigma x}{n}$

d f degrees of freedom

s^2. variance, $s^2 = \frac{\Sigma(x - \bar{x})^2}{n - 1}$

s d standard deviation, $s\,d = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$

ε: standard error, $\varepsilon = \frac{s.\,d}{\sqrt{n}}$

RVAR residual variance, $RVAR = \frac{1}{n-2} \sum \left[w_i - \gamma(1 - e^{-\lambda t_i}) \right]^2$

(indices for time of measurement)

The 95 per cent confidence interval for the unknown mean value $= \bar{x} \pm t \cdot \varepsilon_{\bar{x}}$

The t value is found in Fisher's tables for the given number of degrees of freedom

t test, $t = (\bar{x}_1 - \bar{x}_2) \sqrt{\frac{(n_1 + n_2 - 2) \cdot n_1 \cdot n_2}{(n_1 + n_2)[\Sigma(x_1 - \bar{x}_1)^2 + \Sigma(x_2 - \bar{x}_2)^2]}}$

with d f $= n_1 + n_2 - 2$

The probability, p, of obtaining a t-value at least as great as the calculated *one for a given number of d f is found in Fischer's and Yates' tables.*

If p is greater than 0 05 the difference is said to be non-significant

If p is between 0 01 and 0 05 the difference is said to be almost significant (*)

If p is between 0 001 and 0 01 the difference is said to be significant (**)

If p is 0 001 or less the difference is said to be highly significant (***)

Table 3 Schedule for the sacrificing of mice for whole-body autoradiography at different times after the *administration of labelled* substance Bold figure indicates that the mouse was given the substance by inhalation

Time after administration	Number of mice sacrificed		
	Female	Male	Pregnant female
5 min	3	3	2
20 min	3	1+1	2
1 hour	2+1	1	2
4 hours	2	1	1+1
16 hours			2
24 hours	1	1	1
4 days	1		1
16 days	1+1	1	
Sum of mice	15	9	12

From the block, containing the other half of the mouse, sagittal whole-body sections (20 μ and 80 μ) at different levels were cut in a freezing-room (– 15° C) according to the Ullberg technique To obtain whole sections, an adhesive tape (No 810, Minnesota Mining and Manufacturing Co) was applied to the section surface before cutting

The sections, adhered to the tape, were allowed to dry for 2 days at – 15° C in order to evaporate volatile, labelled compounds Then autoradiograms were made by appositing the sections against X-ray films For 20 μ thick sections Structurix (Gevaert) and for 80 μ thick sections Kodirex (Kodak) were used In order to avoid artefacts due to the melting of fat at room temperature, all dark-room work, except developing, was carried out at – 15° C The distribution patterns obtained from the sections were regarded as representing only non-volatile labelled metabolites of dimethyl mercury

After exposure (10—60 days) the films were separated from the blocks and sections, respectively The films were developed and the plane surface of the blocks photographed on Kodachrome colour slides and the sections stained with hematoxylin and eosin and mounted on glass slides in Eurapal® (Flatters & Garnett Ltd , Manchester, England)

Statistical methods

Conventional statistical methods were applied (Fischer & Yates, 1949, Fischer, 1950, Dixon et Massey, 1951)

The following symbols and formulae were used

x	single observation
n	number of observations
$\bar{x}$	mean of x $\bar{x} = \frac{\Sigma x}{n}$
d f	degrees of freedom
s^2	variance $s^2 = \frac{\Sigma(x - \bar{x})^2}{n - 1}$
s d	standard deviation $s\,d = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$
ε	standard error $\varepsilon = \frac{s\,d}{\sqrt{n}}$

RVAR residual variance $RVAR = \frac{1}{n-2} \sum \left[w_i - x(1 - e^{\;\;n_i}) \right]^2$

(indices for time of measurement)

The 95 per cent confidence interval for the unknown mean value $= \bar{x} \pm t\ \varepsilon_{\bar{x}}$

The t value is found in Fisher s tables for the given number of degrees of freedom

t test $t = (\bar{x}_1 - \bar{x}_2) \sqrt{\frac{(n_1 + n_2 - 2)\ n_1\ n_2}{(n_1 + n_2)[\Sigma(x_1 - \bar{x}_1)^2 + \Sigma(x_2 - \bar{x}_2)^2]}}$

with d f $= n_1 + n_2 - 2$

The probability p of obtaining a t value at least as great as the calculated one for a given number of d f is found in Fischer s and Yates' tables.

If p is greater than 0 05 the difference is said to be non significant.

If p is between 0 01 and 0 05 the difference is said to be almost significant (*)

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Chapter IV. Determination of the retention of methyl mercury in the body, regulated by the sum of the excretion functions

The aim of the investigation was to confirm the assumption, based on observations described in Chapter III (p 42) that the elimination of methyl mercury from the body, regulated by the sum of the excretion functions may be regarded as a reaction of the first order, i e that the number of mercury atoms excreted after an injection of methyl mercury hydroxide at each moment is proportional to the number of atoms retained within the body at any given time

Comments on materials and methods

Twenty five female mice were given ^{203}Hg labelled methyl mercury hydroxide intravenously The dose given to each mouse was 12 μg of mercury and the activity 0 8 μCi The mice were sacrificed 6 days (8 animals) 12 days (9 animals) and 18 days (8 animals) after the injection The radioactivity of each mouse was determined by whole body measurement immediately after the injection and immediately before sacrificing

At the time of sacrifice the mice were exsanguinated in order to avoid contamination of the fur with blood in the further treatment The skins of the dead mice were stripped off and thoroughly cleaned from fat and connective tissue by scraping with a knife

The activity of the skin with fur was determined by using the whole body counting equipment The radioactivity in the body of the mouse was calculated as the difference between the activity of the living mouse and the activity of the skin with fur

The retained amount of mercury in the mouse before sacrificing and in the dead mouse without skin and fur was calculated as per cent of the administered dose

Results and discussion

The whole body retention values and the retention values for the body without skin and fur at the different times of survival are presented in fig 12 It will be seen that the retention function valid for the body without skin and fur to a large extent resembles a straight line in a semi logarithmic

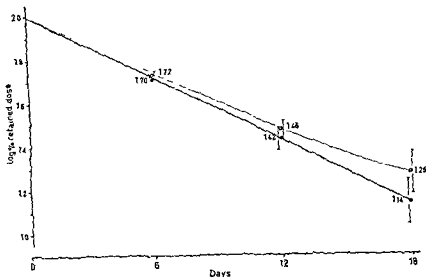


Fig 12 Retention of radiomercury in mice at different times after injection of methylradiomercury The data are given as mean for 8, 9 and 8 mice, respectively, and plotted in the logarithmic scale against survival time Circles represent the means of whole-body counting values Points represent the means of the radioactivity in the body without skin and fur The 95 % confidence limits for the mean values are indicated The dotted line represents an approximate retention curve obtained from the whole-body measurement data and the unbroken line is a straight line connecting the zero-point and the 18 day point for the mice without skin and fur

diagram It is also seen that the retention function, obtained by whole-body counting deviates from this straight line, depending on the increasing amount of radiomercury in the skin and the fur

The data of retention of methyl mercury compounds, obtained from different investigations, using different methyl mercury salts, are probably directly comparable, since different methyl mercuric salts dissociate in the body and the anion has probably no significance for the excretion mechanism (Hughes 1957, Ulfvarson, 1962, Swensson & Ulfvarson, 1963)

Compared to other organic mercuric compounds and inorganic ionised mercury, alkyl mercury compounds are excreted slowly in different species (e g Friberg, 1959, Swensson et al, 1959 a, Swensson & Ulfvarson, 1968 a, 1968 b) This seems partly to be due to an assumed stability of the bond between the mercury and the organic radical a stability suggesting a simple excretion mechanism (Ulfvarson, 1962)

It is a well-known fact that mercury compounds, like several other metals (Jervis et al, 1962) are built in to hairs in man (Stock, 1940, Hoshino et al, 1966), in rats (Gage, 1964, Berglund, 1969), in mice (Back-

Chapter IV. Determination of the retention of methyl mercury in the body, regulated by the sum of the excretion functions

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Results and discussion

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Chapter V. Some factors influencing the radiomercury retention in mice after a single injection of methylradiomercury

[illegible] f the investigation was to study the influence of certain factors [illegible]

[illegible]

1 The influence of sex
2 The influence of the dose of stable methyl mercury
3 The influence of physical activity
4 The influence of a *larger amount of stable methyl mercury hydroxide given six days before a* trace dose of methylradiomercury

The measurement procedure, the mathematical treatment of measurement data and the *symbols used are described in* Chapter III (p 39 ff)

Part I Influence of sex

Comments on materials and methods

Ten female mice (Group D) and ten male mice (Group F) were intravenously given ^{203}Hg labelled methyl mercury hydroxide, dose level D (p 28), i e a dose of 5 mg of mercury per kilo body weight The whole-body radioactivity was measured within one hour after the injection and then every 24th hour during the first 33 days and thereafter at increased intervals in accordance with the indications in fig 13 a and fig 13 b

Results and discussion

The essential data from the measurements are presented in fig 13 a and *13 b as logarithmic* percentages of the retained dose (log % retained dose, p 41) at each measuring time The figures also present the retention functions, valid for the body without fur, obtained from the mean of the $T_{1/2}$ values for the mice within the groups (p 43) The mean of $T_{1/2}$ was for the female mice 12 6 days and for the male mice 12 9 days A statistical comparison between the groups is *presented in table 4, showing that the* difference in biological half life was not significant ($0\,9 > p > 0\,8$) at the dose levels used Miller & Csonka (1968) found a significant difference

strom, 1969), and, as observed by Tejning in 1965, to feathers in birds (Berg et al, 1965 b, 1966, Tejning, 1967 e) The mercury built in to hairs of mice is removed from the body, withdrawn from the circulation and must then, in accordance with generally accepted physiological laws be regarded as excreted from the metabolic point of view

The excretion of methyl mercury in man is closely proportional to the concentration of mercury in the whole body as studied experimentally (Ekman et al, 1968) It is obvious that the mercury content of the hair in man has no significant influence on the total excretion rate, when studied over a period corresponding to about one biological half life of methyl mercury Consequently, the principles of total excretion mechanisms seem to be of similar character in mice and man

Similar mechanisms have been shown to be relevant for rats (Ulfvarson 1962) and poultry (Swensson & Ulfvarson, 1968 a)

For the description of excretion of methyl mercury in rats, the same authors (Swensson & Ulfvarson, 1968 b) used a formula according to which the excretion of mercury compounds at each moment is proportional to the square of the simultaneous concentration in the whole body In this investigation (1968 b), however, no attempts were made to examine the mercury content of the skin and fur

In mice, as seen from the present experiment the retention function of radiomercury, as it is regulated by the sum of the excretion mechanisms, including the transfer to the fur, resembles a straight line in the semilogarithmic scale Thus the excretion of mercury per time unit during the first weeks after a single injection of methyl mercury in mice, at the dose level used, may be regarded as approximately proportional to the mercury concentration in the body at any given time The significance of this conclusion for the mathematical treatment of measurement data was further discussed in Chapter III

	Group D (♀)	Group E (♂)
n	10	10
$T_{1/2}$ mean, days	12 6	12 9
s d, days	±4 1	±5 5
t test	t = 0 157 0 9 > p > 0 8	

Table 5 Doses of stable methyl mercury as mg Hg per kilo body weight, given to the mice used in the experiments described in part 2, Chapter V and in Chapter VI

Group	Level of dosage	Dose Hg per kilo body weight
A	A	0 03 mg
B	B	0 3 mg
C	C	1 0 mg
D	D	5 0 mg

between two different strains of mice in the retention of methyl mercury after a single injection In chickens strain differences in retention of phenyl mercury and inorganic mercury salts were reported by Miller et al (1959 a, 1959 b 1967) No reports of investigations on sex variations in the metabolism of methyl mercury in mice have been found in the literature In quails Backstrom (1969) found some distinct differences between the sexes in the distribution of methyl mercury in the body, but the significance of these differences for the retention of mercury is not known Miller et al (1959 b) found no differences between the sexes in chickens as concerned the retention of phenyl mercury and inorganic mercury salts

Part 2 Influence of the amount of stable methyl mercury

Comments on materials and methods

Forty female mice, divided into four equal groups, were intravenously given ^{203}Hg labelled methyl *mercury hydroxide at four different* levels of dosage in accordance with table 5

Group D in this experiment was identical with group D in the preceding experiment.

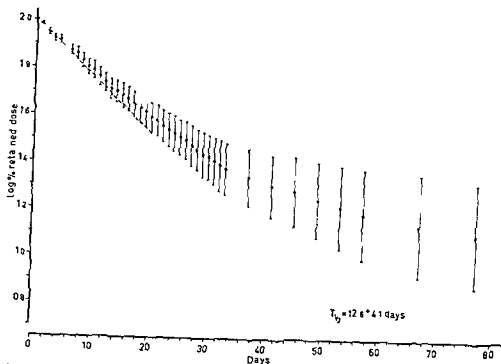


Fig 13 a

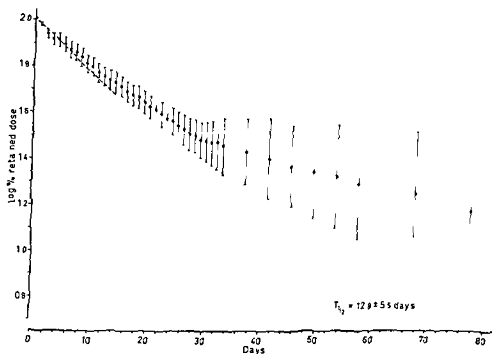


Fig 13 b

Fig 13 a b Retention of radiomercury in female mice (fig 13 a) and male mice (fig 13 b) after an injection of methylradiomercury Means of log % retained dose and 95 % confidence intervals for ten mice in each group are given Dotted lines represent calculated retention functions for the body without skin and fur $T_{1/2}$ values given as mean ± s d

	Group D (♀)	Group E (♂)
n	10	10
$T_{1/2}$ mean days	126	129
s d days	±41	±55
t test		t=0 157 09>p>08

Table 5 Doses of stable methyl mercury as mg Hg per kilo body weight given to the mice used in the experiments described in part 2 Chapter V and in Chapter VI

Group	Level of dosage	Dose Hg per kilo body weight
A	A	0 03 mg
B	B	0 3 mg
C	C	1 0 mg
D	D	5 0 mg

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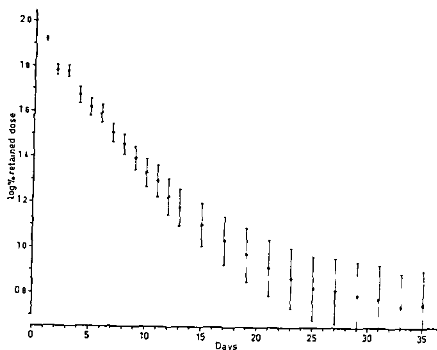


Fig 14 a

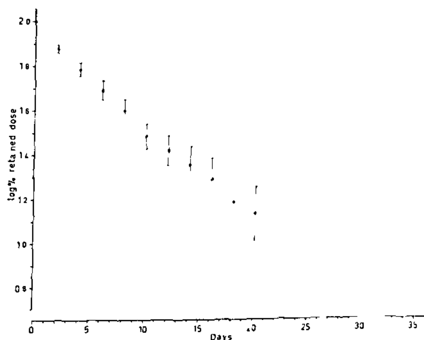
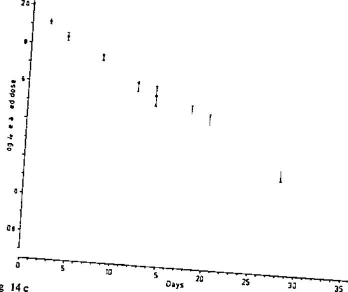
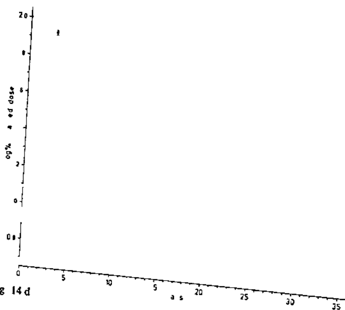


Fig 14 b

Fig 14 a, b, c, d Retention of radiomercury in mice after administration of the same dose of activity in different doses of stable MeHgOH Doses in mg Hg per kilo body-weight Group A (fig 14 a) 0 03, group B (fig 14 b) 0 3, group C (fig 14 c) 1 0 and group D (fig 14 d) 5 0 Means of log % retained dose and 95 % confidence intervals for ten mice in each group are given


20
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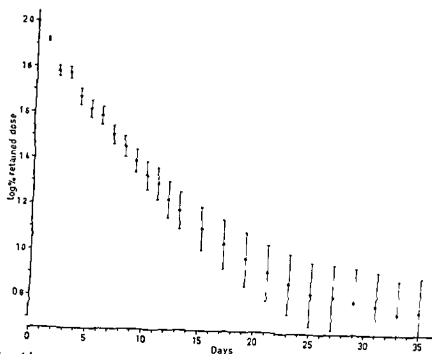


Fig 14 a

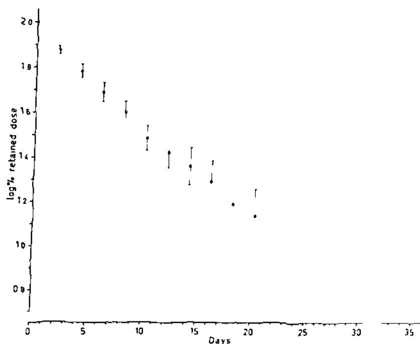


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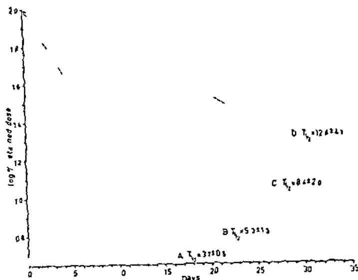


Fig 15 Calculated retention functions valid for body without skin and fur for the mice in groups A B C and D (see fig 14) Values of biological half-life given as mean ± s d

showing that the excretion rate in the species was independent of the total dose given However Berglund (1967) suggested that the differences in excretion rate obtained in different experiments with methyl mercury in rats may have been due to differences in the doses of mercury used

It appears that no definite differences have been proved to occur in man as regards the retention of methyl mercury at different dose levels. However comparison of data from different investigations is rendered difficult due to dissimilarities in materials used and methods employed In trace dose experiments in man Ekman et al (1968) found the $T_{1/2}$-value to be 70 days (mean of three males) as calculated from whole body measurement data Miettinen et al (1969 a) obtained a $T_{1/2}$ value of 78 days (mean of six females plus nine males) by calculation from whole body measurement data in trace-dose experiments in man using methyl mercury biologically incorporated into fish muscle protein In the same investigation Miettinen et al (1969 a) obtained values of biological half life of 47 days (mean of five males) and 130 days (one woman) when calculations were based on the mercury contents of whole blood Calculations on mercury contents in blood from three Japanese cases of methyl mercury poisoning in man gave a mean value of $T_{1/2}$ of about 60 days (Skerfving 1969 Personal communication)

It seems possible that the decreased excretion rate at higher doses which has been observed in the present investigation may be due to a successively increasing mercurial inhibition of sulphydryl enzymes in

Values of mercury concentration in blood originates from Professor Y Tsubaki Brain Research Institute University of Niigata Niigata Japan

The whole body radioactivity of each mouse was measured within one hour after the injection and thereafter at intervals of one to four days according to the indications in figures 14 a b c and d

From five mice taken at random from group D histological examinations were carried out on the following organs the brain including cerebellum and medulla oblongata spinal cord Nervus ischiadicus liver kidney and the myocardium

Results and discussion

No clinical symptoms of methyl mercury poisoning could be observed in the mice given the largest dose of the compound (5 mg Hg per kilo body weight) Nor were any histological alterations seen in the examined tissues from these mice Thus we may conclude that the dose given was not large enough to cause any damage to the tissues which could influence the results of the experiments

The main data from the measurements are given in figures 14 a b c and d as whole body retention functions (log % retained dose versus time) It will be seen from the figures that a considerable difference in the whole body retention of radiomercury occurred between the four groups After 20 days the retained percentage of the dose was in mean 8 9 13 8 25 1 and 38 9 % in the four groups given 0 03 0 3 1 0 and 5 0 mg Hg per kilo body weight respectively

The difference in excretion rate is clearly seen from fig 15 showing the four calculated retention functions corresponding to the four levels of dosage used and valid for the body without skin and fur The values of biological half life obtained from these functions are statistically compared in table 6 As seen from fig 15 and table 6 the biological half life of methyl mercury in the NMRI mice used in this investigation was found to be dependent on the total dose of stable methyl mercury given When the dose was 0 03 mg Hg per kilo body weight the $T_{1\ 2}$ was 3 7 days $\pm$ 0 5 days (mean $\pm$ s d) and when the dose was 5 0 mg Hg per kilo body weight the $T_{1\ 2}$ was 12 6 days $\pm$ 4 1 days the difference being statistically highly significant The doses 0 3 and 1 0 mg Hg per kilo body weight resulted in intermediate values of biological half life (mean 5 3 days and 8 4 days respectively)

In the literature several investigations are reported concerning the retention of methyl mercury in different species e g in man (Ekman et al 1968 Miettinen et al 1969 a) in dogs (Swensson et al 1959 a) in rats (Friberg 1959 Swensson et al 1959 b Swensson & Ulfvarson 1967 Berglund 1969) in mice (Miller & Csonka 1968) in poultry (Tejning & Vesterberg 1964 Tejning 1967 a 1967 c) and in fishes and seals (Tillander 1969) In these investigations the influence of the total dose of methyl mercury given seems to have been studied only in fishes (Tillander 1969)

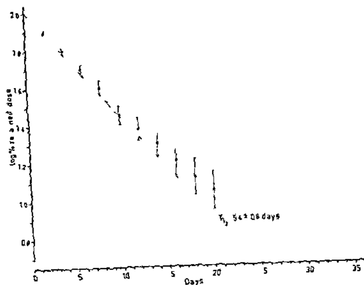


Fig. 16 a

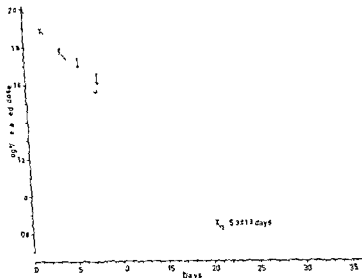


Fig. 16 b

Fig. 16 a, b. Retention of radiomercury in mice with a high degree of physical activity (group F, fig. 16 a) and in control mice (group B, fig. 16 b) after injection of methylradiomercury. Means of log % retained dose and 95 % *confidence* intervals for ten mice in each group are given. Dotted lines *represent* calculated retention functions for the body without skin and fur. Values of biological half life are given as mean ± s.d.

Table 6 *Statistical comparison of calculated biological half lives of radiomercury in mice, given the same dose of radioactivity in different doses of stable MeHgOH (in accordance with table 5)*

Group	A	B	C	D
n	10	10	10	10
$T_{1/2}$ mean, days	3 7	5 3	8 4	12 6
s d , days	±0 5	±1 3	±2 0	±4 1
D	t=6 859 p= <0 001	t=5 378 p= <0 001	t=2 888 0 02>p>0 01	
C	t=7 191 p= <0 001	t=4 155 p= <0 001		
B	t=3 617 0 01>p>0 001			

volved in multiple excretion mechanisms, since mercury compounds are known to be powerful inhibitors of sulphydryl enzymes (e g Fildes, 1940, Barron & Kalnitsky, 1947, Boyer, 1959)

Another explanation of the phenomenon can be derived from the type of bonds established between the mercury compound and sulphydryl groups and maybe other groups in the tissues In Chapter VI (p 76) it is suggested that mercury was primarily attached to "first class sites" Thus, most of the mercury will be bound to first class sites when small doses are given and the second class sites will be to an increasing degree utilized when larger doses are given

The obtained increase in biological half-life when larger doses of stable methyl mercury were given may be due to the fact that the mercury bound to second class sites (third class sites, etc) for some reason is not as easily released from the tissue as the mercury bound to first class sites If that is the case these stronger bonds should cause a slower excretion procedure when larger doses are given and an increasing amount of second class sites are occupied This hypothesis is supported by the findings in the experiment described on p 65 ff Other explanations are also conceivable but it does not seem possible to decide from the present knowledge which mechanisms are responsible for the significantly dose dependent changes in the biological half-life of methyl mercury in mice

Part 3. Influence of physical activity

Comments on materials and methods

Twenty female mice were intravenously injected with ^{203}Hg labelled methyl mercury hydroxide at dose level B (0 3 mg Hg per kilo body weight) Ten

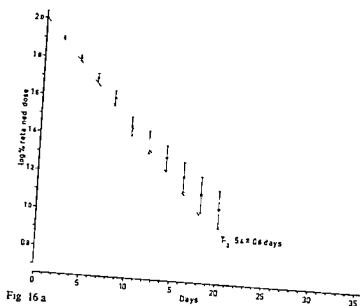


Fig 16 a

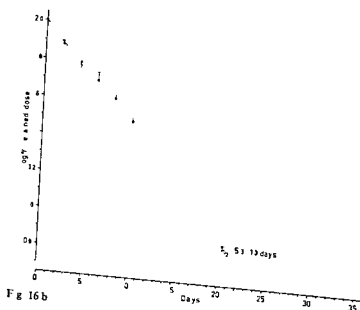


Fig 16 b

Fig 16 a b Retention of radiomercury in mice with a high degree of physical activity (group F fig 16 a) and in control mice (group B fig 16 b) after injection of methylradiomercury Means of log % retained dose and 95 % confidence intervals for ten mice in each group are given Dotted lines represent calculated retention functions for the body without skin and fur Values of biological half life are given as mean ± s d

Table 7 Calculated $T_{1/2}$ for radiomercury and found SGOT-values of the mice in groups B and F with statistical comparison between the groups The mice of group F swam for 45 minutes a day Group B constituted control group

	Group B	Group F
n	10	10
$T_{1/2}$ mean days	5 3	5 4
s d , days	± 1 3	± 0 6
t test	t = 0 307 0 8 > p > 0 7	
n	8	8
SGOT value, mean	123	152
s d	± 29 2	± 57 4
t test	t = 1 217 0 3 > p > 0 2	

of the mice (Group F) were subjected to physical excercise by swimming, as described in Chapter III (p 31) The remaining ten mice, group B, were identical with the mice of group B in the experiment described in part 2 of this chapter

The whole body activity of each mouse was measured within one hour after the injection and then at intervals according to the indications in fig 16 a and fig 16 b

At the end of the experimental period, SGOT-tests were performed on blood samples from eight mice in each group From five mice in each group, chosen at random, muscle samples from the posterior part of the thighs were removed after sacrificing and submitted to histological examination

Results and Discussion

The histological examination did not reveal any structural differences between the muscular tissue samples from the mice of the two groups Nor were any significant differences in the SGOT-values for the mice of the two groups seen, (table 7) though the mean value was somewhat higher for the mice in group F It may be concluded that the physical activity accomplished was of physiological magnitude and did not cause any note worthy pathological alterations influencing the results of the experiments

The essentials of the measurement data are presented in fig 16 a and fig 16 b as whole-body retention functions and calculated retention func tions valid for the body without skin and fur It will be seen that the excretion course of radiomercury was practically the same in the mice

with a high degree of physical activity as in the mice of the control group The calculated biological half life of radiomercury for the mice with a high degree of physical activity was 5 4±0 6 days and for the control animals 5 3±1 3 days (mean ± s d) As seen from the statistical comparison in *table 7 this difference was not significant.*

Observations on the excretion rate of methyl mercury in mice kept singly in metabolism cages (see p 79) had caused one to suspect that a change in *the physical activity might have an influence* on the mercury retention course

Ekman (*1968 Personal communication) observed a slight decrease in* the excretion rate of methyl mercury in a human volunteer when daily participation in a physical training programme was interrupted

In experiments with hens and rats respectively using ^{137}Cs Ekman (1961) and Edwardsson et al (1964) observed a higher initial uptake and a faster elimination of the substance in muscles with a greater physical activity compared to muscles with a lower physical activity

In the present investigation with the physical activity accomplished by swimming no significant change in the excretion rate could be observed It is possible that the accomplishment of a higher degree of physical activity would result in an influence on the excretion rate of methyl mercury

Part 4 Influence of a larger amount of stable methyl mercury hydroxide given six days before a trace dose of methylradiomercury

Comments on materials and methods

Ten female mice (Group G) were intravenously injected with unlabelled *methyl mercury hydroxide at dose level D (5 mg Hg* per kilo body weight) Six days later they were intravenously given ^{203}Hg labelled methyl mercury hydroxide at dose level A (0 03 mg Hg per kilo body weight) At the same time ten previously untreated female mice (Group A) were given the same dose of methylradiomercury *Group A was identical with group* A in the experiment described in part 2 of this chapter

Whole body activity *measurements of the mice were performed* within one hour after the administration of the labelled compound and then at intervals *according to the indications in* fig 17 a and fig 17 b

Results and discussion

The main data from the measurements are presented in fig 17 a and fig *17 b in the* same manner as in the preceding experiments It will be seen *that the retention curves* are very similar for the two groups in spite of the

5 -

Table 7 Calculated $T_{1/2}$ for radiomercury and found SGOT values of the mice in groups B and F with statistical comparison between the groups The mice of group F swam for 45 minutes a day Group B constituted control group

	Group B	Group F
n	10	10
$T_{1/2}$ mean days	5 3	5 4
s d , days	± 1 3	± 0 6
t test	t = 0 307 0 8 > p > 0 7	
n	8	8
SGOT-value, mean	123	152
s d	± 29 2	± 57 4
t test	t = 1 217 0 3 > p > 0 2	

of the mice (Group F) were subjected to physical excercise by swimming as described in Chapter III (p 31) The remaining ten mice, group B, were identical with the mice of group B in the experiment described in part 2 of this chapter

The whole-body activity of each mouse was measured within one hour after the injection and then at intervals according to the indications in fig 16 a and fig 16 b

At the end of the experimental period, SGOT-tests were performed on blood samples from eight mice in each group From five mice in each group, chosen at random, muscle samples from the posterior part of the thighs were removed after sacrificing and submitted to histological examination

Results and Discussion

The histological examination did not reveal any structural differences between the muscular tissue samples from the mice of the two groups Nor were any significant differences in the SGOT values for the mice of the two groups seen, (table 7) though the mean value was somewhat higher for the mice in group F It may be concluded that the physical activity accomplished was of physiological magnitude and did not cause any note worthy pathological alterations influencing the results of the experiments

The essentials of the measurement data are presented in fig 16 a and fig 16 b as whole-body retention functions and calculated retention func tions valid for the body without skin and fur It will be seen that the excretion course of radiomercury was practically the same in the mice

with a high degree of physical activity as in the mice of the control group. The calculated biological half life of radiomercury for the mice with a high degree of physical activity was 5.4 ± 0.6 days and for the control animals 5.3 ± 1.3 days (mean ± s.d.). As seen from the statistical comparison in table 7, this difference was not significant.

Observations on the excretion rate of methyl mercury in mice, kept singly in metabolism cages (see p. 79) had caused one to suspect that a change in the physical activity might have an influence on the mercury retention course.

Ekman (1968, Personal communication) observed a slight decrease in the excretion rate of methyl mercury in a human volunteer when daily participation in a physical training programme was interrupted.

In experiments with hens and rats, respectively, using ^{137}Cs, Ekman (1961) and Edwardsson et al. (1964) observed a higher initial uptake and a faster elimination of the substance in muscles with a greater physical activity compared to muscles with a lower physical activity.

In the present investigation, with the physical activity accomplished by swimming, no significant change in the excretion rate could be observed. It is possible that the accomplishment of a higher degree of physical activity would result in an influence on the excretion rate of methyl mercury.

Part 4 Influence of a larger amount of stable methyl mercury hydroxide given six days before a trace dose of methylradiomercury

Comments on materials and methods

Ten female mice (Group G) were intravenously injected with unlabelled methyl mercury hydroxide at dose level D (5 mg Hg per kilo body-weight). Six days later they were intravenously given ^{203}Hg labelled methyl mercury hydroxide at dose level A (0.03 mg Hg per kilo body weight). At the same time ten previously untreated female mice (Group A) were given the same dose of methylradiomercury. Group A was identical with group A in the experiment described in part 2 of this chapter.

Whole body activity measurements of the mice were performed within one hour after the administration of the labelled compound and then at intervals according to the indications in fig. 17 a and fig. 17 b.

Results and discussion

The main data from the measurements are presented in fig. 17 a and fig. 17 b in the same manner as in the preceding experiments. It will be seen that the retention curves are very similar for the two groups in spite of the

fact that the mice in group G were *simultaneously metabolising an amount* of methyl mercury considerably larger than the *amount of mercury in the* labelled dose The dose of unlabelled methyl mercury *given to the mice* in group *G* was the same as the dose of stable methyl mercury *given to* group D in the experiment described at p 57 ff The obtained *calculated biological* half lives for radiomercury in the groups *A* G *and D are* statistically compared in table 8 It will be seen that the *difference between* group A and group G was almost significant ($T_{1/2}$ mean 3 7 days and 4 2 *days respectively*) and the difference between groups G and D statistically *highly significant* ($T_{1/2}$ mean 4 2 days and 12 6 days respectively)

Calculated from the retention curve obtained from the $T_{1/2}$ value of *group D* (p *60*) the mice of group G at the time of injection of the labelled *compound* had an amount of stable mercury in the body from the first *injection* corresponding to the dose 3 7 mg Hg per kilo body weight

It is of interest to note that the injected dose of methylradiomercury in these mice gave a shorter biological half life ($4\ 2 \pm 0\ 4$ *days mean* $\pm$ *s d*) *than the* $T_{1/2}$ value obtained from the mice given *1 0 mg Hg per kilo* body weight (group C p 60 $8\ 4 \pm 2\ 0$ days) and even shorter than the $T_{1/2}$ *value obtained* from the mice given 0 3 mg Hg per kilo body weight (group *B* p 60 $5\ 3 \pm 1\ 3$ days)

This result suggests that practically no mixing between the two dif *ferent doses* with molecular exchange at the tissue sites occurred This conclusion agrees with results from extraction experiments in vitro show *ing that* it was not possible to release more than a few per cent of trace amounts of labelled methyl mercury bound to tissue homogenates by *flushing* with an excess of unlabelled methyl mercury salts (Ostlund un *published* observation) It also agrees with findings by Friberg (1959) in an experimental study on rats He found that the metabolism of a given *dose of* methylradiomercury was not influenced by subsequent administra *tion of non* radioactive inorganic mercury or a methyl mercury salt

These observations indicate that the bonds established between methyl mercury and tissues are of a different character than the bonds between inorganic mercury and tissues since extraction experiments in vitro have revealed that the main part of a trace dose of inorganic ^{203}Hg can be released without difficulty from a tissue homogenate by flushing with an excess of unlabelled inorganic mercury (Ostlund unpublished observa tion) Similar flushing mechanisms as for inorganic mercury in vitro have been shown to occur in vivo for different substances such as stron tium (Spencer et al 1963) and selenium (Jacobsson & Lindberg 1968)

The retention functions obtained in this experiment compared to the retention function obtained from group D in the experiment described on p 57 ff support the hypothesis for the binding mechanism of methyl mer cury presented on p 76 According to this an increased number of "second class sites are occupied by methyl mercury when larger doses are admin

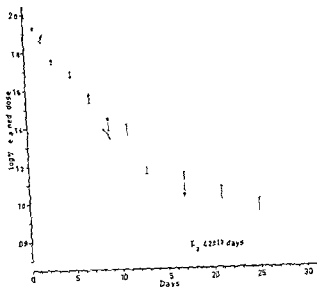


Fig 17 a

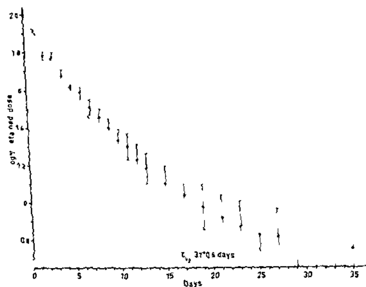


Fig 17 b

Fig 17 a b Retention of radiomercury in mice after an injection of a trace dose of methylradiomercury Group G (fig 17 a) was simultaneously metabolising a large dose of unlabelled MeHgOH given six days before the injection of the labelled compound Group A (fig 17 b) constituted control group Means of *log %* retained dose and 95 % confidence intervals for ten mice in each group are given Dotted lines represent calculated retention functions for the body *without skin* and for $T_{1/2}$-values are given as mean ± s d

Table 8 Calculated biological half life of radiomercury for the mice in groups A G and D with statistical comparison between the groups A and G and G and D, respectively The mice of group G were given a dose of stable methyl mercury (5 mg Hg per kilo body weight) six days before the injection of labelled methyl mercury (dose of stable mercury 0 03 mg per kilo body weight) Group A and D are identical with groups A and D in the experiment, described in part 2, Chapter V

	Group A	Group G	Group D
n	10	10	10
$T_{1/2}$ mean, days	3 7	4 2	12 6
s d, days	±0 5	±0 4	±4 1
t test	$t = 2\ 252$ $0\ 05 > p > 0\ 02$		$t = 6\ 477$ $p < 0\ 001$

istered, and the release of mercury from the tissue occurs more slowly from these sites than from the first class sites Thus, when the large dose of unlabelled methyl mercury was given to the mice of group G, first class sites and to a large extent, second class sites were occupied During the six days before the injection of the labelled compound, a certain part of the mercury left the body, and primarily the mercury attached to the first class sites

Thus, when the trace dose of labelled methyl mercury was given, it would be mainly bound to the liberated first class sites, and consequently be metabolised in the same way as a trace dose of methyl mercury, independent of the formerly administered large dose of methyl mercury hydroxide

Chapter VI. Distribution of radiomercury between certain tissues at different levels of dosage of stable methyl mercury hydroxide

The aim of the experiment was to investigate whether the distribution of radiomercury between different tissues after a single intravenous injection of methyl^{203}Hg mercury hydroxide is dependent on the dose of stable mercury given

Comments on materials and methods

Forty female mice divided into four equal groups H, I, K and L were intravenously given 1 μCi of ^{203}Hg labelled methyl mercury hydroxide Solutions of the four different specific activities described in Chapter III (table 2 p 29) were used for the different groups to obtain four levels of dosage of stable mercury so that the mice of group H were given 0 03, group I 0 3 group K 1 0 and group L 5 0 mg Hg per kilo body weight (dose levels A B C and D respectively)

Six days after the injection the whole body radioactivity of the mice was measured and the mice sacrificed after blood collection The following tissues were selected for radioactivity determination Blood, blood plasma, liver kidneys brain and skeletal muscular tissue

The activity per ml of blood corpuscles was calculated from the activity (cpm) per ml blood (C_B), per ml plasma (C_P) and the hematocrit value (H %) according to the definition of the hematocrit value

$$\text{Activity in cpm per ml blood corpuscles} = \frac{100\,C_B + H\ C_P - 100\,C_P}{H}$$

For each sample the activity per gram tissue was calculated as percentage of the activity per gram animal* at the time of sacrifice (% gram dose per gram tissue)

* To obtain the activity which the whole mouse should have given when counted in the gamma spectrometer used for sample measurement the whole body measurement value for the mouse was multiplied by a factor corresponding to the quotient between the counting efficiencies of the gamma spectrometer and the whole body counting apparatus This quotient was obtained by measuring four animals injected with ^{203}Hg labelled MeHgOH in the whole body counter and then homogenising them (Ultraturrax TP 18/2) and measuring the homogenate in the gamma spectrometer

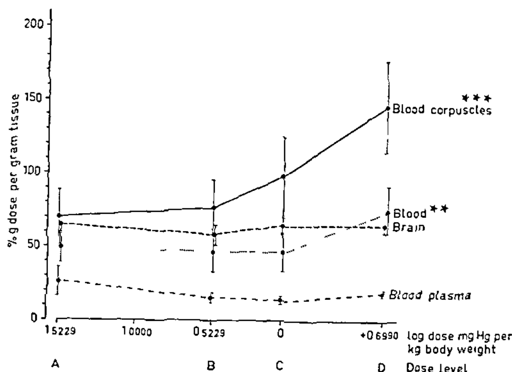


Fig 18 Radiomercury contents of blood, blood corpuscles blood plasma and brain six days after injections of four different doses of stable methylradiomercury in mice The doses given were in mg Hg per kilo body weight 0 03 (dose level A) 0 3 (dose level B), 1 0 (dose level C) and 5 0 (dose level D) The ordinates give the contents per gram tissue as percentages of the dose retained per gram animal at time of sacrifice In each point the mean for ten mice and the 95 % confidence interval are given Asterisks indicate the statistical significance between dose level A and dose level D

Results

The concentration of ^{203}Hg in the selected tissues, given as % gram dose per gram tissue, is plotted in fig 18 and fig 19 against the logarithmic value of the dose of stable mercury

For blood corpuscles, muscular tissue and liver a statistical comparison between the four groups with t-test values is given in tables 9 10 and 11 The quotients in radiomercury concentration between blood cells and blood plasma and between brain tissue and blood are statistically compared in tables 12 and 13

It will be seen from fig 18 that the concentration of mercury in blood rises more than the average concentration in the body when the dose is increased from level A to level D, the difference being significant It is also seen that the concentration in the blood corpuscles rises still more the difference A—D being highly significant (table 9)

No statistically significant differences in the % gram dose per gram tissue were seen in the plasma concentration The quotient between con centration of mercury in blood corpuscles and concentration in blood

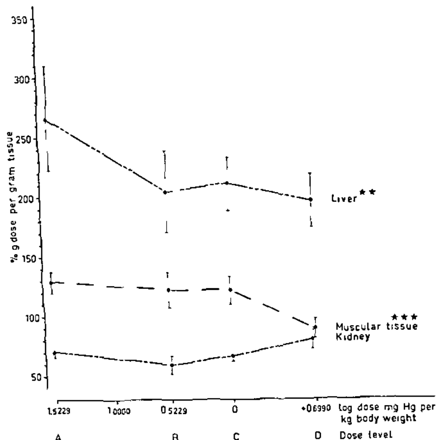


Fig 19 Radiomercury contents of liver, muscular tissue and kidneys six days after injection of four different doses of stable methylradiomercury in mice The values are presented in the same manner as the values in fig 18 The kidney values are multiplied by the factor 10^{-1} Asterisks indicate the statistical significance between dose level A and dose level D

plasma rises when larger doses of methyl mercury are given As seen from table 12 the difference in quotient between dose level A and D was significant

In the brain the accumulation seemed to be practically constant at the different doses given (fig 18) Thus, since the uptake in blood corpuscles increases with increasing dose of mercury the brain blood ratio is dose-dependent the difference between dose level A and dose level D being statistically significant (table 13)

The uptake in skeletal muscular tissue was not proportional to the dose given It will be seen from fig 19 and table 10 that the uptake is lower at level D than at level A, the difference being statistically highly significant

Table 9 Radiomercury concentration in blood corpuscles six days after administration of the same dose of radioactivity in different doses of stable methyl mercury hydroxide in mice Concentration given as activity per gram tissue as percentage of the activity per gram animal at the time of sacrifice

Level of dosage	A	B	C	D
n	10	10	10	10
Concentration, mean	69 8	76 2	98 0	146 2
s d	± 25 9	± 25 2	± 37 8	± 43 1
D	t = 4 767	t = 4 198	t = 2 632	
	p < 0 001	p < 0 001	0 05 > p > 0 02	
C	t = 1 933	t = 1 448		
	0 1 > p > 0 05	0 2 > p > 0 1		
B	t = 0 541			
	0 6 > p > 0 5			

Table 10 Radiomercury concentration in muscular tissue six days after administration of the same dose of radioactivity in different doses of stable methyl mercury hydroxide in mice Concentration given as activity per gram tissue as percentage of the activity per gram animal at the time of sacrifice

Level of dosage	A	B	C	D
n	10	10	10	10
Concentration mean	129 6	121 2	120 3	87 9
s d	± 12 4	± 19 0	± 16 4	± 11 2
D	t = 7 890	t = 4 707	t = 5 146	
	p < 0 001	p < 0 001	p < 0 001	
C	t = 1 427	t = 0 113		
	0 3 > p > 0 2	p > 0 9		
B	t = 1 148			
	0 3 > p > 0 2			

The accumulation in the liver (fig 19 and table 11) shows a change between different doses similar to that in muscular tissue and the difference was significant

In the kidneys the uptake did not differ significantly between the dose levels compared to the average of the animal, although a tendency of increased uptake at level D can be seen from fig 19

Table 11 Radiomercury concentration in liver tissue six days after administration of the same dose of radioactivity in different doses of stable methyl mercury hydroxide in mice Concentration given as activity per gram tissue as percentage of the activity per gram animal at the time of sacrifice

Level of dosage	A	B	C	D
n	10	10	10	10
Concentration, mean	266 7	202 9	208 8	193.2
s d	±60 3	±43 9	±30 0	±30 6
D	t=3 367 0 01>p>0 001	t=0.556 0 6>p>0 5	t=1 141 0 3>p>0 2	
C	t=2 719 0 02>p>0 01	t=0 342 0 8>p>0 7		
B	t=2 554 0 02>p>0 01			

Discussion

The distribution of methyl mercury compounds in the body is fairly well known from several investigations In man, it has been studied by chemical and histochemical analysis of clinical and autopsy material (e g Hook et al 1954, Kurland et al, 1960, Takeuchi et al, 1962, Tejning, 1968 a, 1968 b) and by experimental studies using radioisotopes (Ekman et al, 1968, Miettinen et al 1969 a)

The distribution of methyl mercury has been thoroughly studied by experiments in laboratory animals, i e dogs (Swensson et al, 1959 a, Yoshino et al 1966), cats (Yamashita, 1964), rabbits (Swensson, 1952, Friberg et al 1957, Swensson et al, 1959 a, Berlin, 1963), rats (Friberg, 1959, Swensson et al, 1959 a, 1959 b, Ulfvarson, 1962, Gage, 1964, Yoshino et al, 1966, Swensson & Ulfvarson, 1967, 1968 b, Moriyama, 1968), birds (Tejning & Vesterberg, 1964, Tejning, 1967 a, 1967 b, 1967 c, 1967 d, 1967 e, Swensson & Ulfvarson, 1968 a, Backstrom, 1969) and fishes (Hannerz, 1968, Backstrom, 1969, Miettinen et al., 1969 b, Ohmomo et al, 1969)

In mice the distribution of methyl mercury is known from whole body autoradiographic studies (Berlin & Ullberg, 1963, Ukita, 1967, Backstrom, 1969) and other investigations using radioisotopes (Suzuki et al, 1961, 1963)

These investigations have revealed that the distribution of methyl mercury differs in several respects from the distribution of other organic mercury compounds and inorganic mercury, and that the distribution of methyl mercury shows great similarities between different species

The aim of this investigation was not to study the distribution of methyl mercury in particular, but to elucidate whether there are any differences in the mercury distribution if different single doses of methyl mercury are given to mice No investigations concerning this particular question have been found reported in the literature, though some experiments with phenyl mercury in rats indicate that the amount of substance given may have an influence on the distribution of this compound (Laug & Kunze, 1949) Ohmomo et al (1969)observed a difference in distribution velocity between methylradiomercury with and without carrier when administered to pikes Ulfvarson (1962) concluded, from experiments with long term exposure in rats, that alkyl mercury compounds distribute between different organs according to a simple distribution equilibrium

In this investigation, certain distinct differences in the distribution were seen, when different doses of methyl mercury were given

The uptake of methyl mercury in blood was not proportional to the dose given, but increased between dose levels C and D (the difference C—D as well as A—D being significant) This difference was mainly due to a considerable increase in the uptake in blood corpuscles at higher doses, (A—D highly significant), since no significant differences in the plasma uptake could be seen between the different doses given Thus, the blood corpuscle plasma quotient increased with an increasing dose of administered mercury and the difference in quotient between dose A and dose D was significant (table 12)

At all dose levels used, the uptake in the blood corpuscles was higher than in the plasma This observation agrees with results from investigations in man (Tejning, 1968 a, 1968 b, Ekman et al 1968) rabbits (Swensson et al, 1959 a, Berlin, 1963), rats (Ulfvarson 1962, Gage 1964, Takeda et al, 1968 a), birds and fishes (Backstrom, 1969), showing that methyl mercury in common with several other organic mercury compounds and in contrast to inorganic mercury is transported in blood mainly bound to the blood corpuscles and to a smaller extent to the blood plasma (Swensson et al 1959 b, Ulfvarson 1962 Takeda et al 1968 a) Swensson et al., (1959 a) and Ekman et al (1968) studied the blood corpuscle blood plasma quotient at different times after a single dose of methyl mercury in rabbits and dogs and in man respectively They found the quotient to be fairly constant during the observation period in all three species However, in these experiments, the observation periods were too short to cause any considerable decrease in the total mercury content in the body and consequently the results are not contradictory to the findings in mice where the distribution of methyl mercury between blood cells and plasma was significantly dependent on the amount of substance given

The explanation of this phenomenon may have a connection with the observations by Takeda et al, (1968 b), concerning the binding of ethyl mercury to the erythrocytes The authors showed that ethyl mercury was

Table 12 Quotient between concentration of radiomercury in blood corpuscles and in plasma six days after administration of the same dose of radioactivity in different doses of stable methyl mercury hydroxide in mice

Level of dosage	A	B	C	D
n	10	10	10	10
Mean	3.39	5.17	7.31	8.30
s d	±2.12	±1.66	±2.91	±3.43
D	$t=3.849$ $0.01>p>0.001$	$t=2.483$ $0.05>p>0.02$	$t=0.694$ $0.5>p>0.4$	
C	$t=3.442$ $0.01>p>0.001$	$t=1.938$ $0.1>p>0.05$		
B	$t=2.015$ $0.1>p>0.05$			

Table 13 Quotient between concentration of radiomercury in the brain and in the blood six days after administration of the same dose af radioactivity in different doses of stable methyl mercury hydroxide in mice

Level of dosage	A	B	C	D
n	10	10	10	10
Mean	1.47	1.54	1.56	0.93
s d	±0.46	±0.79	±0.53	±0.27
D	$t=3.215$ $0.01>p>0.001$	$t=2.296$ $0.05>p>0.02$	$t=3.323$ $0.01>p>0.001$	
C	$t=0.400$ $0.7>p>0.6$	$t=0.070$ $p>0.9$		
B	$t=0.230$ $0.9>p>0.8$			

easily transferred into the nonstromal part of the erythrocytes through the stroma. On the other hand ethyl mercury once combined with hemoglobin was not easily transferred through the stroma. If the same reaction is valid for methyl mercury, an initial uptake in the erythrocytes would cause a high blood corpuscle: plasma quotient when equilibrium between blood plasma and other tissues is maintained. However, the initial uptake in the erythrocytes should be proportional to the dose given, unless the time required for establishing the distributional equilibrium is longer when larger doses of mercury are given.

Since no data concerning the time required for establishing an equilibrium in the distribution of methyl mercury in the body are available, it can only be assumed that the above mentioned mechanism of binding to hemoglobin may be responsible for the observed differences in the blood corpuscle plasma quotient at different doses if the time needed to reach the equilibrium is dependent on the total dose given

Another explanation of the phenomenon may be the occurrence of sulphydryl groups with different degrees of affinity to the methyl mercury ion as discussed in connection with the uptake in muscular tissue (below) A combination of both discussed mechanisms is also possible

The uptake in muscular tissue was not proportional to the dose given, but decreased when the dose was increased the difference between the lowest and the highest doses being highly significant (fig 19, table 10)

The methyl mercury ion as well as other mercury compounds is known to have an extremely high affinity to animal protein and especially to the sulphydryl groups (e g Hughes et al, 1950, Perkins, 1952, 1953, 1961, Hughes, 1957, Passow et al, 1961, Benesch & Benesch, 1962) The decreased uptake in muscular tissue which was observed when higher doses were given may be due to an initial saturation of available sites for the compound and thus a lack of free groups with affinity for methyl mercury It seems reasonble to assume that different tissue sites are to different degrees available to methyl mercury and that the affinity of the methyl mercury may be of varying intensity toward different tissue sites depending on various factors e g their stereochemical position (For a review of such factors, see e g Åkerfeldt 1964) If we assume that this is the case, we may grade the sulphydryl groups of protein as first class sites second class sites etc, according to falling affinity (availability) to methyl mercury In this discussion for the sake of simplicity the sites are only divided in two classes, referred to as first and second class sites Then with increasing doses of the compound a decreased uptake will be seen in tissues where first class sites are becoming consumed as long as other tissues still have an excess of first class sites provided that no barriers circumscribe the mobility of the mercury compound within and between the tissues This explanation seems more plausible than the above mentioned saturation theory" since a lack of free sites in a tissue after administration of a certain dose of methyl mercury should result in a ceased uptake in the tissue at higher doses and not in a gradually changed uptake as seen in blood plasma in the present investigation The theory based on the assumption of the existence of sites with different affinity to methyl mercury is also supported by the changes in uptake seen in the liver (fig 19 and table 11) where the decrease in concentration compared to the average of the animal is greatest between doses A and B and the concentration fairly constant between doses B and D

In the kidney, no significant differences could be seen in the uptake

between the different levels of dosage, though a tendency to increased uptake was seen at level D

Besides the theories of explanation given above, this tendency may be due to a potentially occurring increased breakdown of the compound to inorganic mercury when larger amounts of substance are given, with a following accumulation of inorganic mercury in the kidney (Berlin & Ullberg, 1963) It can also be seen as an attempt by the body to increase the renal excretion of the compound when the level of dosage is raised

For the brain, the uptake seemed to be practically proportional to the dose given. As seen from fig 18 the curve for the brain uptake is almost parallell to the curve for the plasma concentration No significant differences in the brain plasma quotient were found at the different dose levels Swensson et al (1959 b) found a constant ratio between the blood and the brain in rats at different times after subacute administration of a methyl mercury compound and suggested a simple distribution equilibrium In the present investigation, the difference in the ratio brain blood in mice between different levels of dosage was significant (table 13) Suzuki et al (1963) also observed an increasing brain blood ratio in mice at different survival times after a single injection of a methyl mercury salt These results however, do not necessarily contradict the result by Swensson et al (1959 b) since the observation period in their investigation was three weeks, during which time the change in the animals' total mercury contents may have been too low to cause any considerable change in the erythrocyte plasma quotient Thus in such an experiment, the blood brain ratio will remain practically constant, even if the same distribution laws are valid for rats as for mice Gage (1964) concluded, from experiments with a methyl mercury salt in rats that the concentration in plasma was approximately equal to that in the brain

In the mice used in this investigation and within the levels of dosage used there seems to be a simple distribution equilibrium for methyl mercury between blood plasma and the brain

Since no data concerning the time required for establishing an equilibrium in the distribution of methyl mercury in the body are available, it can only be assumed that the above mentioned mechanism of binding to hemoglobin may be responsible for the observed differences in the blood corpuscle plasma quotient at different doses if the time needed to reach the equilibrium is dependent on the total dose given

Another explanation of the phenomenon may be the occurrence of *sulphydryl* groups with different degrees of affinity to the methyl mercury ion, as discussed in connection with the uptake in muscular tissue (below) A combination of both discussed mechanisms is also possible

The uptake in muscular tissue was not proportional to the dose given, but decreased when the dose was increased, the difference between the lowest and the highest doses being highly significant (fig 19, table 10)

The methyl mercury ion as well as other mercury compounds is known to have an extremely high affinity to animal protein and especially to the sulphydryl groups (e g Hughes et al, 1950, Perkins, 1952, 1953, 1961, Hughes, 1957, Passow et al, 1961, Benesch & Benesch, 1962) The decreased uptake in muscular tissue which was observed when higher doses were given may be due to an initial saturation of available sites for the compound and thus a lack of free groups with affinity for methyl mercury It seems reasonble to assume that different tissue sites are to different degrees available to methyl mercury and that the affinity of the methyl mercury may be of varying intensity toward different tissue sites depending on various factors, e g their stereochemical position (For a review of such factors, see e g Åkerfeldt, 1964) If we assume that this is the case, we may grade the sulphydryl groups of protein as first class sites, second class sites etc, according to falling affinity (availability) to methyl mercury In this discussion, for the sake of simplicity, the sites are only divided in two classes referred to as first and second class sites Then, with increasing doses of the compound a decreased uptake will be seen in tissues where first class sites are becoming consumed as long as other tissues still have an excess of first class sites, provided that no barriers circumscribe the mobility of the mercury compound within and between the tissues This explanation seems more plausible than the above mentioned "saturation theory" since a lack of free sites in a tissue after administration of a certain dose of methyl mercury should result in a ceased uptake in the tissue at higher doses and not in a gradually changed uptake as seen in blood plasma in the present investigation The theory, based on the assumption of the existence of sites with different affinity to methyl mercury is also supported by the changes in uptake seen in the liver (fig 19 and table 11) where the decrease in concentration compared to the average of the animal is greatest between doses A and B and the concentration fairly constant between doses B and D

In the kidney, no significant differences could be seen in the uptake

ter et al, 1940, Swensson, 1952, Hagen, 1955) The reverse, an excretion of mercury via the respiratory system after administration of methyl mercury does not seem to have been studied, although Ekman et al (1968) concluded from their excretion studies in man, that there can be no other significant routes of excretion than urine and feces After injection of inorganic, labelled mercury in rats Clarkson & Rothstein (1964) observed a certain exhalation of radiomercury

The results of the present experiment show that, if exhalation of radiomercury in mice after intravenous injection of methylradiomercury actually does occur, it must be considered *quantitatively negligible*

Part 2 Excretion via urine and feces

Comments on materials and methods

Sixteen female mice were intravenously given ^{203}Hg labelled, "twice purified" MeHgOH (Chapter 1, p 17) The activity given to each mouse was 2 μCi and the amount of mercury 6 μg, corresponding to the dose 0 3 mg Hg per kilo body weight The excretion products were collected and the radioactivity measured as described in Chapter III (p 31—33 and p 35, respectively) The amount of ^{203}Hg excreted in feces and urine was calculated as per cent of the given dose and from these values the retained percentage of the dose after each day was calculated At the time of collecting excreta the radioactivity of each mouse was measured by the whole-body counting technique The two mice in each metabolism cage were regarded as a unit and the retained percentage of the given dose was calculated for the unit at every measuring time The experiment was interrupted after 14 days for eight of the animals (four units) and after 21 days for the rest of the animals

Results

The main excretion occurred via feces (ca 40 % of the dose in 7 days and ca 70 % in 21 days) and the fecal excretion was fairly high during the whole sampling period (fig 20) The urinary excretion was fairly high during the first day and then diminished (fig 21) The excreted percentage of the dose after 7 days was 14 % and after 21 days ca 17 % The retention curve for ^{203}Hg calculated from the urinary and fecal excretion values (fig 22) was almost identical with the retention curve obtained by whole body counting measurement (fig 23)

Discussion

Pilot experiments had indicated that the excretion rate was slower when the mice were kept singly compared to the excretion rate when the mice

Chapter VII. Excretion routes of ^{203}Hg after intravenous injection of methyl mercury hydroxide

The whole-body measurement investigations (Chapter V) after injection of methylradiomercury have shown the retention of ^{203}Hg in the body, as it is regulated by the excretion of the labelled compound The aim of this investigation was to study the routes by which the radiomercury leaves the body of the mouse The investigation was divided in two parts, one for the study of exhalation of radiomercury and one for the study of the renal and fecal excretion

Part 1. Excretion by exhalation

Comments on materials and methods

Three female mice were intravenously given 0 2 ml of a water solution of ^{203}Hg-labelled methyl mercury hydroxide The activity given to each mouse was 2 μCi and the amount of mercury corresponded to the dose 4 mg Hg per kilo body-weight Immediately after the injection the mice were placed in the polyethylene container of the air-sampling equipment described in Chapter III (p 33—35), and the sampling was started The collecting tubes were exchanged every half hour during the first seven hours after the injection, when the sampling was interrupted and the radioactivity of the contents in the collecting tubes measured

Results

In all collecting tubes the radioactivity was below the detection limit of the method of determination (see Chapter II, p 27) That means in this case, that the exhaled and collected radioactivity every half hour after the injection constituted less than 0 0003 % of the given dose

Discussion

Inhalation of methyl mercury compounds is known to be of importance as a way of uptake in man (e g Hunter et al , 1940, Dinnman et al , 1958, Freyschuss et al , 1958) and has been studied in animal experiments (Hun

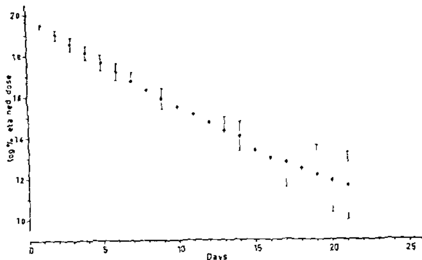


Fig 22 Retention of radiomercury as log % retained dose in the mice from which feces and urine were collected Means and 95 % confidence intervals for retention values *as calculated from obtained* values *for fecal* and *urinary excretion* The values for the first fourteen days are for sixteen mice (mathematically treated as eight units) and for the remaining period for eight mice (four units)

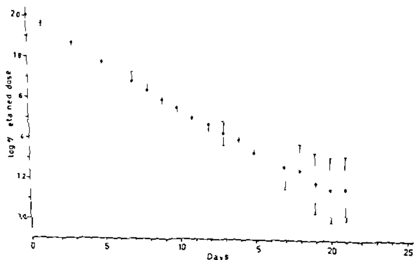


Fig 23 Retention of radiomercury as log % retained dose in the mice from which feces and urine were collected Means and 95 % confidence intervals for retention values obtained by whole body radioactivity measurements The values for the first fourteen days are for sixteen mice (mathematically treated as eight units) and for the remaining period for eight mice (four units)

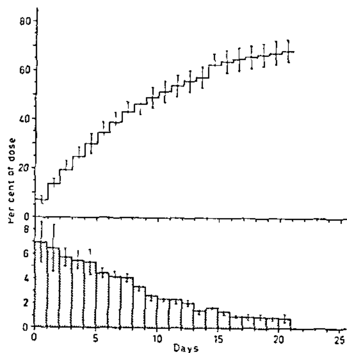


Fig 20 Daily and accumulated excretion of ^{203}Hg in feces after administration of methylradiomercury Ordinates indicate means and 95 % confidence intervals The values for the first fourteen days are for sixteen mice (mathematically treated as eight units) and for the remaining period for eight mice (four units)

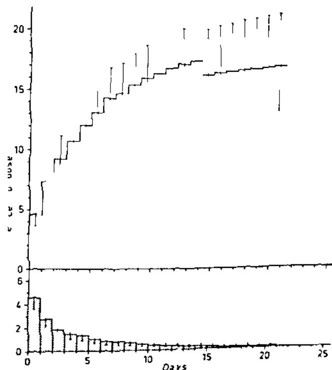


Fig 21 Daily and accumulated excretion of ^{203}Hg in urine after administration of methylradiomercury in mice Ordinates indicate means and 95 % confidence intervals *The values for the first fourteen* days are for sixteen mice (mathematically treated as eight units) and for the remaining period for eight mice (four units)

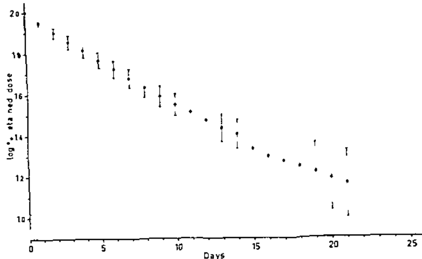


Fig 22 Retention of radiomercury as log % retained dose in the mice, from which feces and urine were collected Means and 95 % confidence intervals for retention values as calculated from obtained values for fecal and urinary excretion The values for the first fourteen days are for sixteen mice (mathematically treated as eight units) and for the remaining period for eight mice (four units)

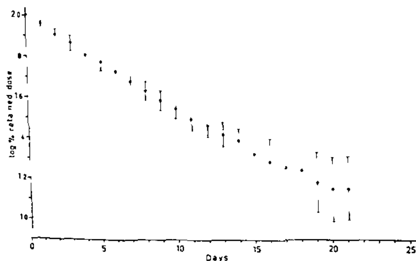


Fig 23 Retention of radiomercury as log % retained dose in the mice from which feces and urine were collected Means and 95 % confidence intervals for retention values obtained by whole body radioactivity measurements The values for the first fourteen days are for sixteen mice (mathematically treated as eight units) and for the remaining period for eight mice (four units)

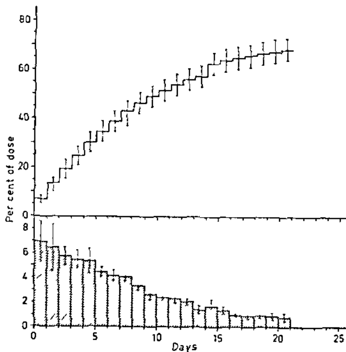


Fig 20 Daily and accumulated excretion of ^{203}Hg in feces after administration of methylradiomercury in mice Ordinates indicate means and 95 % confidence intervals The values for the first fourteen days are for sixteen mice (mathematically treated as eight units) and for the remaining period for eight mice (four units)

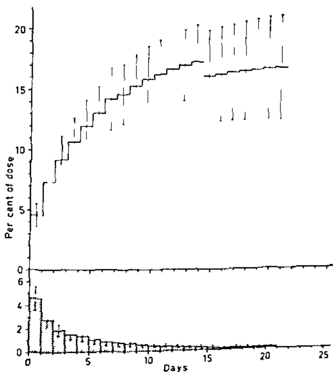


Fig 21 Daily and accumulated excretion of ^{203}Hg in urine after administration of methylradiomercury in mice Ordinates indicate means and 95 % confidence interval The values for the first fourteen days are for sixteen mice (mathematically treated as eight units) and for the remaining period for eight mice (four units)

Chapter VIII. Extractions and thin-layer chromatographic investigations on selected tissues and excreta after injections of methylradiomercury

The aim of the investigation was to study the chemical form in which ^{203}Hg is present in different organs and in feces and urine after a single injection of ^{203}Hg labelled methyl mercury hydroxide in mice

Comments on materials and methods

For extractions from tissues five female mice were intravenously given ^{203}Hg labelled MeHgOH The dose of mercury was 5 mg Hg per kilo body weight. The activity given was for three mice 5 μCi and for two mice 30 μCi The mice given 5 μCi were exsanguinated and sacrificed 24 hours 6 days and 14 days after the injection From the mouse killed 24 hours after the injection the blood, the liver, the kidneys, the brain and muscular tissue were collected for further treatment From the mice killed after 6 days and 14 days the livers and kidneys were collected The mice given 30 μCi were exsanguinated and sacrificed 6 days and 14 days after the injection From these mice, the blood and the brains were harvested for examination

For extractions from excreta two female mice provided with the urine collection equipment according to Pope (p 33) were intravenously given 25 μCi of ^{203}Hg labelled MeHgOH The dose given to each mouse was 50 μg of mercury, corresponding to the dose 2 5 mg Hg per kilo bodyweight The mice were placed one by one in metabolism cages

The feces and urine from the 1st 3rd and 6th days and the feces from the 14th day were submitted to further treatment, the urine from the 12th 13th and 14th days was pooled for the examination

The tissues and excreta collected were homogenised and each homogenate divided into two equal parts In one part, the extractable percentage of the radiomercury was determined according to extraction method 3 (p 37) The other part of the homogenate from each tissue and excreta, respectively was extracted according to method 1 (p 36) and thin layer chromatography of the benzene extracts was performed according to method A (p 21)

Results

The extractability of the radiomercury in the different tissues according to the method used are listed in table 14

were kept in groups of five as was the case in the whole body counting experiments (Chapter V) This observation was considered as a result of the very limited physical activity in a mouse kept alone in a cage Thus in this experiment the mice were kept two by two in the metabolism cages in order to maintain a certain physical activity in the animals and the two mice were regarded as a unit in all mathematical treatment of measurement data

The occurrence of a building in process of mercury to the hairs of the fur was shown and discussed in Chapter IV However this phenomenon did not interfere with this investigation since the fur was measured together with the rest of the animal during the whole body counting The turnover of hairs was considered negligible during the experiment Further more the potential radioactivity of shed hairs was not lost but measured together with the feces Thus the congruency between the retention curves obtained by whole body counting and by calculation from excretion values shows that with exception of the fur the urine and feces are the only significant routes of excretion of ^{03}Hg after a single injection of methyl radiomercury in mice This conclusion agrees with the fact that no radio activity was found in the exhalation air after injection of methylradio mercury (see above) and is also in accordance with the findings in man (Ekman et al 1968)

In the present investigation on mice the main route for excretion of ^{03}Hg was found to be feces 70 % of the given dose had left the body via feces in 21 days It is of interest to note that the greatest daily excretion occurred during the first 24 hours after the administration of the sub stance in spite of the fact that the route of administration was intravenous injection

Feces has been shown to be the main route of elimination of mercury after administration of methyl mercury also in other animals (Friberg 1959 Gage & Swan 1961 Ulfvarson 1962 Gage 1964 Yamashita 1964) and in man (Ekman et al 1968 Miettinen et al 1969 a)

In these species the renal excretion is low compared to the fecal excre tion In the mice in this investigation the renal excretion though lower than the fecal excretion was considerable especially during the first days after administration (fig 21) After 21 days ca 17 % of the dose was collected in the urine and the value was 12 % as early as five days after administration The urinary excretion decreased continuously with the time after injection and no tendency of increase in the excretion as observ ed in man (Ekman et al 1968) was seen

occurred in the form of methyl mercury The possibility that the unextractable fraction of the radiomercury was an organic metabolite of methyl mercury may be excluded, since every plausible organic mercury compound should have been extracted, at least in part, by the extraction methods used The conclusion must be that no other organic mercury compound is formed in vivo from methyl mercury administered to mice The character of the unextractable part of the mercury in the tissues is discussed further in Chapter IX

Table 14 Per cent extractable radiomercury in certain tissues according to extraction method 3 (p 37) at different times after i v injection of methylradiomercury in mice

Time of survival	Tissue	Extractable radiomercury
24 hours	Brain	98 0 %
	Blood	95 2 %
	Liver	95 0 %
	Kidney	95 6 %
	Muscle	96 7 %
6 days	Brain	96 9 %
	Blood	96 7 %
	Liver	94 8 %
	Kidney	96 2 %
14 days	Brain	95 9 %
	Blood	89 6 %
	Liver	93 1 %
	Kidney	92 7 %

It will be seen that in all tissues used and at the different times of survival, the main part of the radiomercury was in the extractable form Thus the value of extractability varied in all determinations between 89 6 % and 98 0 % For the brain, the values after survival times of 24 hours, 6 days and 14 days were 98 0 %, 96 9 % and 95 9 %, respectively The corresponding values were for blood 95 2 %, 96 7 % and 89 6 %, for the liver 95 0 %, 94 8 % and 93 1 %, and for the kidney 95 6 %, 96 2 % and 92 7 %

The applicability of extraction method 3 to excreta has proved to be limited (p 39) and it will only be stated here that the amount of extractable radiomercury in the excreta in no case was below 70 % of the total radio mercury content

The radiochromatograms from benzene extracts of organs and excreta in all cases showed one single organic mercury compound the R_f-value of which coincided with that of methyl mercury No illustrations of these chromatograms will be published here, since *they give no information in* addition to the results in fig 2 (p 23) and fig 44 (p 118)

Discussion

It may be concluded from the results that the main part of the retained mercury in the body after administration of methyl mercury to mice was present in the form of methyl mercury and that the excretion mainly

occurred in the form of methyl mercury The possibility that the un extractable fraction of the radiomercury was an organic metabolite of methyl mercury may be excluded since every plausible organic mercury compound should have been extracted at least in part, by the extraction methods used The conclusion must be that no other organic mercury compound is formed in vivo from methyl mercury administered to mice The character of the unextractable part of the mercury in the tissues is discussed further in Chapter IX

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Discussion

It may be concluded from the results that the main part of the retained mercury in the body after administration of methyl mercury to mice was present in the form of methyl mercury and that the excretion mainly

occurred in the form of methyl mercury The possibility that the unextractable fraction of the radiomercury was an organic metabolite of *methyl mercury may be excluded, since every plausible organic mercury* compound should have been extracted, at least in part, by the extraction methods used The conclusion must be that no other organic mercury *compound is formed in vivo from methyl mercury administered to mice* The character of the unextractable part of the mercury in the tissues is discussed further in Chapter IX

Chapter IX. Pilot experiments concerning the stability of the carbon-mercury bond of methyl mercury in vivo

In Chapter VIII it was stated that the *main part* of administered methyl mercury was present in the form of methyl mercury in the body and that no other organic mercury compounds were formed in vivo No definite conclusions could be made about the chemical form of the remaining unextractable part of the mercury, i e if it occurs in the form of methyl mercury or if the mercury carbon bond has been broken and the mercury occurs in the inorganic form

The aim of this investigation was to obtain some data on the stability of the carbon mercury bond in the methyl mercury ion after intravenous administration of methyl mercury hydroxide to mice

The investigation was divided into three parts Separate descriptions of these experiments will be given here, followed by a general discussion for the three parts

Part 1 Exhalation of $^{14}CO_2$ after intravenous injection of ^{14}C-labelled methyl mercury hydroxide

Comments on materials and methods

Four female mice were intravenously given 1 μCi of ^{14}C labelled MeHgOH in water solution The amount of mercury given to each mouse was 6 μg corresponding to the dose 0 3 mg Hg per kilo body weight Immediately after the injection the mice were placed one by one in the polyethylene container of the air sampling equipment arranged for sampling of carbon dioxide (Chapter III p 34) and the sampling was started

In one of the mice the air was collected for three subsequent periods of 30 minutes after the injection for one period of one hour after 24 hours and for one period of one hour 72 hours after the injection

In the other three mice the air was sampled during the first third fifth seventh and tenth hour after the injection and then after 24 hours 2 3 4 7 and 10 days after the injection each time for a period of one hour

The radioactivity of the collected carbon dioxide was measured and calculated for each period as per cent of the given dose

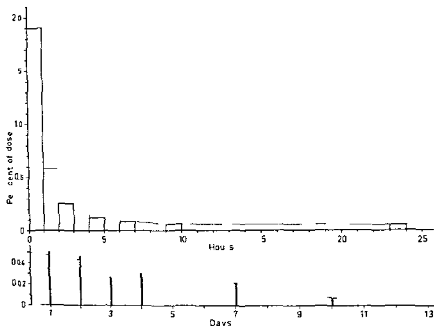


Fig 24 Exhalation of $^{14}CO_2$ as per cent of given dose of radioactivity after injection of ^{14}C-labelled MeHgOH *Unbroken columns represent the mean of three or four mice* broken lines represent estimated data

Results

For the first hour third hour fifth hour etc after the injection the mean of the exhaled percentage of the dose was calculated for the three or four mice respectively To get an approximate estimation of the amount of radioactivity exhaled over a longer period the exhalation of activity in the intervals between the collecting periods was estimated by interpolation Thus the exhaled amount of ^{14}C as $^{14}CO_2$ could be estimated as percentage of given dose to 4 2 % *during the first day* 1 2 % during the second and then 0 9—0 3 % every day during the third to the tenth day During the first three hours after the injection the estimated value was 2 7 % The obtained measurement values and estimated data are graphically presented in fig 24

Part 2 Intravitally induced changes in the ratio $^{14}C/^{203}Hg$ after injection of a mixture of ^{14}C-labelled and ^{203}Hg-labelled methyl mercury hydroxide

Comments on materials and methods

Two female mice were intravenously given a water solution of a mixture of ^{14}C labelled and ^{203}Hg labelled methyl mercury hydroxide *The activity*

Table 15 Results of radiomercury extractions from homogenates of liver and kidneys at different post injection times after administration of $Me^{203}HgOH$ Extractions performed according to method 3 (p 37)

Time of survival		0 min	20 min	1 tim	2 tim	4 tim
Extractable ^{203}Hg in tissue homogenate, %	Liver	96 8	93 8	95 0	94 9	96 3
	Kidneys	97 0	96 1	94 0	95 8	97 0
Estimated amount of the 'inextractable mercury compound" in tissue homogenate, %	Liver	0 2	3 2	2 0	2 1	0 7
	Kidneys	0 0	0 9	3 0	1 2	0 0

of ^{14}C given to each mouse was ca 15 μCi and the activity of ^{203}Hg ca 5 μCi The amount of mercury was 120 μg, corresponding to the dose 6 mg Hg per kilo body-weight

One mouse was sacrificed immediately after the injection and the other mouse 60 minutes after the injection The kidneys and the livers from the mice were homogenised and extracted according to the Gage-Westoo method (Chapter III)

The final benzene extract (called benzene phase) from the liver of the mouse sacrificed 60 minutes after the injection was collected From both mice, the remaining parts of the extracted tissue homogenates (called water phases) were collected after washing twice with equal volumes of benzene

A part of the benzene phase was submitted to thin layer chromatography according to method A and method C (Chapter II)

From the benzene phase and the water phases and the solution used for injection three 500 μl samples of each solution were submitted to determination of gamma activity (gamma spectrometer) Three 100 μl samples of each solution were submitted to determination of beta activity in a liquid scintillation counter The values obtained from the liquid scintillation counting were corrected for activity from ^{203}Hg by a correction factor obtained by counting a standard solution of $Me^{203}HgOH$ in the gamma-spectrometer and in the liquid scintillation counter The ratio between cpm emanating from ^{14}C (corrected value) and cpm from ^{203}Hg per unit volume was calculated for each solution as a mean of the ratios obtained from the triple analysis

Results

The thin layer chromatography revealed that the only detectable labelled compound in the benzene phase was the methyl mercury ion

The mean values for the ratios between cpm from ^{14}C and cpm from ^{203}Hg for the different solutions were, from the mouse sacrificed 60 minutes after the injection

The solution used for injection 3.51
The benzene phase liver 3 50, kidneys 3 48
The water phase liver 0 0, kidneys 0 57

The activity of the water phase from the mouse sacrificed immediately after the injection was too low to permit determination of the quotient with the equipment used

Part 3 Observations on the amount of extractable methyl mercury in selected tissues at different times after a single injection of methyl mercury hydroxide

The aim of the investigation was to study potential changes in the extractable amount of methyl mercury in tissues, due to a suggested initial production of inorganic mercury (see this chapter, Discussion) from injected methyl mercury hydroxide

Comments on materials and methods

Six female mice were intravenously given a water solution of "twice purified" ^{203}Hg labelled MeHgOH The radioactivity given to each mouse was 25 μCi and the amount of mercury 12 μg corresponding to the dose 0 6 mg Hg per kilo body weight One mouse was sacrificed immediately after the injection and the others after 20 minutes, and after one, two and four hours respectively The liver and the kidneys from each mouse were homogenised and extracted according to method 3 (Chapter III, p 37), i e in order to approximately determine the amount of potentially present labelled inorganic mercury or other unextractable mercury compounds All extractions for this determination were made in duplicate from each organ

Results

For each homogenate the extractable amount of radiomercury was calculated as per cent of the total amount of radiomercury As stated in Chapter III the extractable percentage of activity according to the method used after addition of Me^{203}HgOH in vivo or in vitro was 97 3 ± 0 4 % (mean ± s d) Thus an approximate estimation of the percentage of an unextractable mercury compound produced in vivo was obtained by subtracting the experimental values of extractability from the rounded off mean of the reference value (97 %)

The obtained values of extractability as well as the estimated percentages of the unextractable radiomercury at different times of survival are listed in table 15

Table 15 Results of radiomercury extractions from homogenates of liver and kidneys at different post injection times after administration of Me^{203}HgOH Extractions performed according to method 3 (p 37)

Time of survival		0 min	20 min	1 tim	2 tim	4 tim
Extractable ^{203}Hg in tissue homogenate %	Liver	96 8	93 8	95 0	94 9	96 3
	Kidneys	97 0	96 1	94 0	95 8	97 0
Estimated amount of the inextractable mercury compound" in tissue homogenate %	Liver	0 2	3 2	2 0	2 1	0 7
	Kidneys	0 0	0 9	3 0	1 2	0 0

of ^{14}C given to each mouse was ca 15 μCi and the activity of ^{203}Hg ca 5 μCi The amount of mercury was 120 μg, corresponding to the dose 6 mg Hg per kilo body-weight

One mouse was sacrificed immediately after the injection and the other mouse 60 minutes after the injection The kidneys and the livers from the mice were homogenised and extracted according to the Gage-Westoo method (Chapter III)

The final benzene extract (called benzene phase) from the liver of the mouse, sacrificed 60 minutes after the injection was collected From both mice, the remaining parts of the extracted tissue homogenates (called water phases) were collected after washing twice with equal volumes of benzene

A part of the benzene phase was submitted to thin-layer chromatography according to method A and method C (Chapter II)

From the benzene phase and the water phases and the solution used for injection three 500 μl samples of each solution were submitted to determination of gamma activity (gamma spectrometer) Three 100 μl samples of each solution were submitted to determination of beta activity in a liquid scintillation counter The values obtained from the liquid scintillation counting were corrected for activity from ^{203}Hg by a correction factor, obtained by counting a standard solution of Me^{203}HgOH in the gamma-spectrometer and in the liquid scintillation counter The ratio between cpm emanating from ^{14}C (corrected value) and cpm from ^{203}Hg per unit volume was calculated for each solution as a mean of the ratios obtained from the triple analysis

Results

The thin layer chromatography revealed that the only detectable labelled compound in the benzene phase was the methyl mercury ion

The mean values for the ratios between cpm from ^{14}C and cpm from ^{203}Hg for the different solutions were, from the mouse sacrificed 60 minutes after the injection

benzene phase after administration of a mixture of ^{14}C labelled and ^{203}Hg labelled MeHgOH

With the extraction method used the main part of potentially produced inorganic mercury was left in the homogenate which after the extraction procedure was collected and examined No noticeable differences in the quotient $^{14}C/^{203}Hg$ were seen in the extracted methyl mercury compared to the quotient of the administered solution From the preceding experiment with sampling of exhaled $^{14}CO_2$ it was concluded that a certain break up of the mercury carbon bond of methyl mercury did occur in vivo and two alternatives of metabolisation processes were given Of these two alternatives the present result supports the one with a break-down of methyl mercury to inorganic mercury The other mechanism an exchange of methyl groups ought to have resulted in formation of methyl mercury with a lower ratio $^{14}C/^{203}Hg$

If a splitting of the mercury carbon bond did occur the produced inorganic mercury should have been left in the water phases after the extraction procedure As was expected no noticeable activity was found in the water phase remaining after the extraction of the homogenate from the mouse killed immediately after the injection In the animal killed 60 minutes after the injection a certain radioactivity was left in the water phase, indicating the occurrence of an unextractable mercury compound or a change in the character of the mercury tissue bonds, rendering the extraction more difficult Such a change in the bonds does not seem to occur as shown by the experiment in part 3 of this chapter (discussed below) and consequently the activity must be regarded as having arisen from an unextractable mercury compound the most probable one being inorganic mercury This assumption is strongly supported by the $^{14}C/^{203}Hg$ quotient found for the activity in the water phase (0 0 and 0 57 against 3 51 in the injection solution) showing a considerable decrease in ^{14}C activity compared to the activity of ^{203}Hg From these findings and since it seems unlikely that any other unextractable mercury compound than inorganic mercury would be formed from methyl mercury it may be concluded that the radioactivity found in the water phases mainly corresponded to the emergence of inorganic mercury in the tissues This conclusion is in agreement with the theory of a certain break-down of methyl mercury though it cannot be concluded that a break down of the methyl mercury ion was the only source of inorganic mercury It is known from autoradiographic investigations (Berlin & Ullberg 1963) that inorganic mercury after intravenous administration in mice accumulates in the liver and in the kidneys Consequently the occurrence of a radiochemical impurity of $^{203}Hg^{2+}$ in the solution used for injection in the present investigation could have resulted in a relatively large amount of inorganic radio mercury in the examined tissues, even if the percentage of the impurity was very low in the solution used

It will be seen that the amounts of unextractable mercury immediately after the injection were for the liver 0 2 % and for the kidneys 0 % At longer times of survival, the percentage of unextractable radiomercury first increased, having its maximum after 20 minutes for the liver (3 2 %) and after one hour for the kidneys (3 0 %) The amount of unextractable radiomercury then decreased and was after four hours for the liver close to the initial amount (0 7 %) and for the kidneys the same as the initial amount (0 %)

Discussion

The metabolic behaviour of methyl mercury compounds in man and animals, especially the slow rate and uniform course of the excretion process over long periods after administration suggests that no significant decomposition, as regards the carbon mercury bond, occurs in vivo (Ulfvarson, 1962)

Gage & Swan (1961) concluded that a slight decomposition of methyl mercury dicyandiamide occurred after subcutaneous administration in rats

Clarkson et al (1965) observed a considerable intravital break-down of a mercurial diuretic (chlormerodrin) to inorganic mercury in kidney tissue of rats, rabbits and chickens

Backstrom (1969) made observations in autoradiographic studies suggesting an initial break-down of methyl mercury to inorganic mercury after the administration of the compound in mice The phenomenon was visible in the liver and more pronounced in the kidneys

In this investigation, a certain exhalation of radioactive carbon after administration of ^{14}C-labelled MeHgOH was found This could not be due to exhalation of the intact compound, since no radiomercury was found in exhaled air after injection of methylradiomercury (Chapter VII) Nor can the result be explained only by the occurrence of radiochemical impurities in the labelled compound used since the activity exhaled during the first 24 hours constituted about 4 2 % of the dose given and the radio chemical purity of the compound used was determined as being >97 % The conclusion must be that the exhalation of $^{14}CO_2$ at least in part was due to a break up of the carbon mercury bond and oxidation of the methyl group with formation of carbon dioxide This process may be regarded as a break down of the methyl mercury ion to inorganic ionised mercury or as a process of exchange of methyl groups i e in the formation of a new methyl mercury ion in which the organic group has been exchanged with another identical group The exhalation of $^{14}CO_2$ was quantitatively practically negligible after a couple of days, showing that the break up of the mercury carbon bond, only occurred initially and could comprise only a small part of the total dose of methyl mercury given

In the experiment described as part 2 of this chapter methyl mercury was selectively extracted from tissues, then purified and collected in a

administered methyl mercury with a probable production of inorganic mercury

The reason for the retardation of the decomposition reaction is not known It may be due to an inactivation of participating enzymes by the produced inorganic mercury or may be correlated to the establishing of bonds between mercury and tissues preventing the break down of methyl mercury The elucidation of this question will demand further investigations

In the experiment described in part 3 of this chapter methyl mercury was "quantitatively" extracted from the livers and the kidneys of mice at different times after injection of methylradiomercury in order to determine the approximate percentage of unextractable mercury present. The results showed that in all samples at least 93.7 % of the radiomercury could be extracted in the form of methyl mercury. The estimated amount of unextractable mercury was in no case larger than 3.2 %, showing that a potential break down of methyl mercury to inorganic mercury could comprise only a minute part of the administered methyl mercury hydroxide. The amount of unextractable mercury did change with the time after injection in a way which seemed to follow a certain pattern. Thus the extractability first decreased to a minimum within one hour and then increased to reach approximately the initial value after four hours.

The decrease in extractability most probably was due to the occurrence of an unextractable mercury compound. It could hardly be caused by a change in the chemical character of the bond between mercury and tissue, since the extractability four hours after the injection had risen to approximately the initial value, and the formation of a stronger bond, persisting for a couple of hours must be regarded improbable. The observed increasing extractability at survival times 2 and 4 hours seems to be due to an elimination of the unextractable compound from the liver and the kidneys respectively. As shown by the experiment in part 2 of this chapter the unextractable mercury compound formed is very probably the inorganic mercury ion, Hg^{2+}. This assumption also agrees with the fact that no other organic mercury compounds than methyl mercury could be detected in tissues after injection of methylradiomercury (Chapter VIII).

As was stated above it is not possible to conclude that the occurrence of inorganic mercury in the organs was entirely due to its production in the tissues since a similar result may be obtained if a radiochemical impurity of $^{203}Hg^{2+}$ was present in the solution injected in the animals. However, it may be noted that the time for the emergence of inorganic mercury in the liver and the kidneys fairly well corresponded to the time for the break down of methyl mercury as it was indicated by the exhalation of $^{14}CO_2$. It also seems reasonable that an initial production of inorganic mercury in a few tissues should result in a subsequent distribution in the body with a certain disappearance of the compound from the organs where the production took place. Such a disappearance observed in the present investigation, could hardly be explained if the inorganic mercury was a radiochemical impurity, accumulated in the liver and the kidneys after administration.

The conclusion of the three parts of this experiment must be that the mercury-carbon bond of the dominant part of a dose of methyl mercury hydroxide, intravenously given to mice, remains intact. Certain signs indicated the occurrence of an initial decomposition of a small part of

concentration while the concentration in the brain stem and the medulla oblongata was of the same degree as in blood The activity in the spinal cord and in the hypophysis was comparable to that of the liver In the ovaries of a pregnant mouse a moderate uptake was seen with a slight concentration to the walls of larger follicles The corpora luteae did not differ from the rest of the ovary Also in the oviduct a moderate uptake was seen concentrated to the mucosa In pregnant mice the placentae showed a concentration comparable to that of blood No activity could be seen in the fetuses There were no noticeable concentrations in the thymus the testes in bone marrow in muscular or connective tissue or in compact bone

After this time of survival (5 minutes) the activity of the sections representing only non volatile metabolites was too low to give any evaluable autoradiograms

Twenty minutes Twenty minutes after the administration the activity of dimethyl mercury and metabolites was still low in blood and very high in fat tissue (fig 26) An increasing activity was seen in the liver in the kidneys and in the red pulp of the spleen The gall bladder showed the same activity as the liver tissue A fairly high accumulation was seen in the pancreas The activity in bone marrow was considerably lower than that in blood The concentration in the nasal mucosa was very high A high activity was also observed in the mucosa of the oral cavity the pharynx and the oesophagus No considerable changes in other tissues were observed

In autoradiograms representing only non volatile metabolites (fig 30 fig 36) the activities in different organs were the same as for dimethyl mercury plus metabolites except in the following tissues in which practically no activity could be demonstrated All body fat depots and brown fat The central nervous system and the placentae of pregnant mice

One hour The autoradiograms representing dimethyl mercury plus metabolites one hour after administration showed a decreased total activity in the body compared to shorter times of survival and a relatively increased activity in the liver and the kidneys (fig 27) The activity in the kidneys was concentrated to the inner parts of the cortex No difference in activity was seen between the gall bladder and the liver tissue A slightly increased accumulation was seen in the adrenal cortex The activity of fat tissue had decreased in relation to the blood concentration and was of the same magnitude as that of the liver The picture of the nasal the oral the pharyngeal and the oesophageal mucosa was unchanged A marked accumulation occurred in the gastric mucosa and in the mucosa of some intestinal portions A slightly increased concentration was seen in the central nervous system and the hypophysis

Chapter X. Distribution of ^{203}Hg-labelled dimethyl mercury in mice, studied by whole-body autoradiography

Comments on materials and methods

Materials and methods are described in Chapter III

Results

No differences in the distribution patterns could be seen between mice, given dimethyl mercury by inhalation and mice given the substance by intravenous injection, irrespective of time of survival Therefore, in the following, the results will be described independent of route of administration of dimethyl mercury

Initially, the concentration of dimethyl mercury decreased rapidly in the circulating blood and the activity was simultaneously localised mainly to the fat depots The distribution pattern then changed rapidly with increasing time of survival The radioactivity in fat tissue decreased and no activity could be seen there 16 hours after administration At the same time a slow increase of the activity was seen in most other tissues Most marked was the accumulation in the liver, the kidneys the bronchi and the nasal mucosa After longer periods of survival no marked differences between different organs were seen except the kidneys in which a considerable accumulation remained In the following a detailed description of the results at the different times of survival will be given

Five minutes As early as 5 minutes after the administration (fig 25) the concentration was low in the blood and high in all depots of body fat in brown fat, and in tissues containing considerable amounts of lipophilic cells (preputial glands and Harder's gland) The bronchi of the lungs and the nasal mucosa were also sites for a marked accumulation of activity A moderate concentration could be seen in the liver and the kidneys and a fairly high accumulation in the adrenal cortex Concentrations of the same magnitude as found in blood were seen in the lungs (except the bronchi), the spleen and in the intestines A moderate uptake was seen in the parotid gland and the submaxillary and sublingual salivary glands showed a marked concentration

In the central nervous system, the activity varied between different parts Compared to blood, the cerebral cortex and the cerebellum had a lower

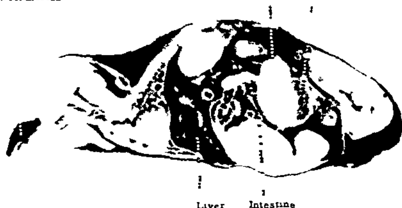


Fig 27 Autoradiogram of ^{203}Hg dimethyl mercury and metabolites in a pregnant mouse 1 hour after inhalation of the compound Note the very high uptake in fat tissue nasal mucosa the bronchi and the liver A certain activity is seen in the placentae though the uptake in the fetuses is very low



Fig 28 Autoradiogram representing ^{203}Hg dimethyl mercury and metabolites 4 hours after an i v injection in a male mouse The activity in fat tissue is fairly low High activity is seen in the liver the kidney the nasal mucosa and in certain parts of the intestine Note the uptake in the oral mucosa The activity in bone marrow and in the brain is low



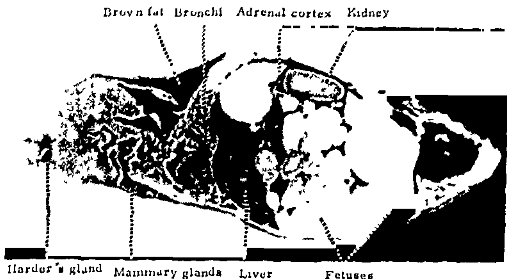


Fig 25 Autoradiogram of a pregnant mouse 5 minutes after an i v injection of ^{203}Hg-*dimethyl mercury* The picture shows the distribution of dimethyl mercury plus metabolites Very high accumulation (white areas) is seen in all fat tissue and in tissues containing lipophilic cells (mammary glands, Harder's gland) Note high accumulation in the bronchi and in the adrenal cortex A certain accumulation is seen in the liver and in the kidney Very low accumulation is seen in the brain and in the fetuses

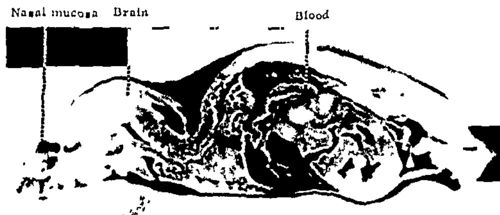


Fig 26 Autoradiogram of ^{203}Hg dimethyl mercury plus metabolites in a female mouse 20 minutes after an i v injection Very high accumulation is seen in all fat tissue in the nasal mucosa and in the bronchi A fairly high uptake is seen in the liver The activity in the blood and in the brain is low

Fig 29 Autoradiogram of ^{203}Hg-dimethyl mercury and metabolites 24 hours after an i v injection of the compound The only organ with considerable activity is the kidney in which an accumulation is seen in the inner part of the cortex

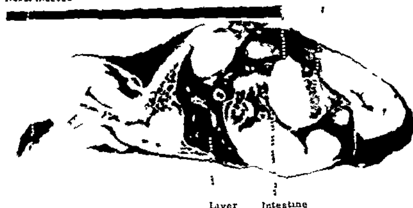


Fig 27 Autoradiogram of ²⁰³Hg dimethyl mercury and metabolites in a pregnant mouse 1 hour after inhalation of the compound Note the very high uptake in fat tissue nasal mucosa the bronchi and the liver A certain activity is seen in the placentae though the uptake in the fetuses is very low



Fig 28 Autoradiogram representing ²⁰³Hg dimethyl mercury and metabolites 4 hours after an i v injection in a male mouse The activity in fat tissue is fairly low High activity is seen in the liver the kidney the nasal mucosa and in certain parts of the intestine Note the uptake in the oral mucosa The activity in bone marrow and in the brain is low



A concentration comparable to that of the blood was seen in the placentae of pregnant mice In the fetuses there was a considerable concentration seen in the nasal, the oral and the oesophageal mucosa The bronchi were sites for a marked accumulation and a moderate uptake was seen in the liver The radioactivity in the remaining fetal tissues was low

No considerable changes in the concentrations of the other maternal organs were observed compared to shorter times of survival

The autoradiograms representing only non-volatile metabolites (fig 32, fig 33) showed a distribution pattern almost identical to the described pattern for dimethyl mercury plus metabolites, except for fat tissue, the central nervous system and the placentae of pregnant mice where no activity could be seen

Four hours Four hours after administration the concentration of radio activity was still somewhat higher in fat tissue than in blood (fig 28) Higher concentrations than in fat tissue were found in the bronchi, the liver, the kidneys and in the adrenal cortex A slight accumulation was seen in muscular tissue A few portions of the intestine showed a marked accumulation, while the concentration in the rest of the intestine was comparable to the concentration in blood Compared to mice with a survival time of 1 hour no further noticeable changes were seen

The distribution pattern representing only non-volatile metabolites (fig 31) corresponded to the described pattern for dimethyl mercury plus metabolites except that practically no concentration was seen in fat tissue or in the central nervous system

Sixteen hours Sixteen hours after administration no differences could be seen between the distribution pattern of dimethyl mercury plus metabolites as obtained from the autoradiograms of the surfaces of the deepfrozen blocks and the pattern of only non volatile metabolites This was the case also for the times of survival, longer than 16 hours

The highest activities were seen in the kidneys (inner part of cortex), the liver, and the nasal mucosa Concentrations of the same degree as in the blood were seen in muscular tissue, the intestines the spleen and in the lungs where no accumulation could be seen in the bronchi The activity in the upper part of the digestive tract had diminished A moderate uptake was seen in the lens of the eye A considerably lower concentration than in blood was seen in fat tissue and in the central nervous system The hypophysis showed a moderate uptake There was no detectable uptake in connective tissue or compact bone

In the fetuses of pregnant mice, the concentration was of the same order as in the maternal blood, and no accumulation was seen in the bronchi or in the nasal, oral or pharyngeal mucosa There was a marked uptake in the lens of the eye (cf fig 35)

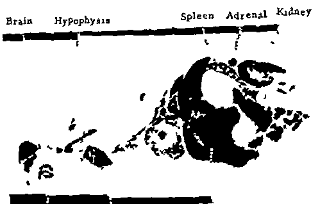


Fig 30 Autoradiogram representing non volatile metabolites of ^{203}Hg dimethyl mercury in a female mouse 20 minutes after an i v injection of the compound High accumulation (white areas) is seen in the liver the bronchi the nasal and oral mucosa and in the hypophysis The activity is marked in the adrenal cortex and the kidney Note the low activity in all body fat and in the brain

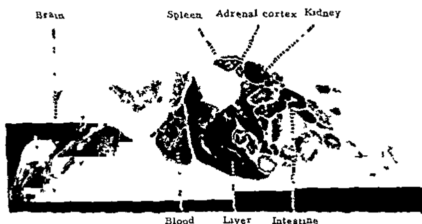


Fig 31 Autoradiogram representing non volatile metabolites of ^{203}Hg dimethyl mercury 4 hours after inhalation in a female mouse Highest uptake is seen in the liver the kidney and in the nasal mucosa Note uptake in oral pharyngeal and oesophageal mucosa in the adrenal cortex and the red pulp of the spleen The activity of the brain is considerably lower than that of blood

Twenty four hours Twenty four hours after administration the only organs containing any considerable degree of activity were, beside the nasal mucosa the kidneys (fig 29) The localisation within the kidneys was the inner part of the cortex The differences between most structures including the fetuses and the blood had almost disappeared and no specific accumulation was seen in the bronchi However lower activity than the

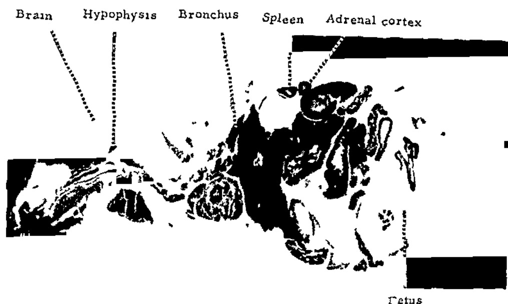


Fig 32 Autoradiogram representing non volatile metabolites of dimethyl mercury in a pregnant mouse 1 hour after i v injection of ^{203}Hg dimethyl mercury Note the uptake (light areas) in the bronchi the adrenal cortex and the nasal, oral and oesophageal mucosa The uptake in fat tissue and in the brain is low

average was seen in the central nervous system and in fat tissue No activity was detectable in connective tissue or compact bone

Four days Four days after the administration (fig 34) the only noticeable change in the distribution pattern compared to the pattern after 24 hours was an increased concentration in the central nervous system in relation to other tissues and an increased uptake in the lens of the eye, both in adult mice and in fetuses of pregnant mice An uptake was seen in the hair follicles of the vibrissae

Sixteen days Sixteen days after the administration of dimethyl mercury the increase of concentration within the central nervous system was still more pronounced and the activity of the brain of the same degree as the activity of the blood A marked uptake of activity in the basal parts of the hairs of the fur was also seen

Discussion

A comparison between autoradiograms from animals killed after different post injection times showed that a considerable part of the administered radioactivity left the body within a few hours The elimination was quite obvious as early as after 1 hour and was still more pronounced after 16 hours This indicates a process of exhalation of radiomercury since no



Fig 33 Detail of fig 32 showing the uptake in the fetal tissues Note the accumulation in the bronchi and in the nasal and oral mucosa

considerable activity could be seen in the kidneys or in the urinary bladder in the early stages of the course This observation and the fact that dimethyl mercury was easily volatilised from whole body sections indicates that dimethyl mercury behaves as a chemically inert substance towards animal tissues Consequently the distribution should occur only according to physical laws and in accordance with its lipophilic nature If that is the case there should be equilibrium on one hand between fat depots and blood and on the other hand between blood and the air in the alveoli of the lungs and the dimethyl mercury should be removed from the body when respiration occurs

As seen from the distribution patterns dimethyl mercury accumulated only in fat tissue and in tissues containing lipids or lipophilic cells The activity seen in other tissues corresponded mainly to the non volatile metabolisation products while no concentration of metabolites could be demonstrated in fat tissue

Most mercury compounds organic and inorganic are known to establish strong bonds with sulphydryl groups in animal protein (e g Hughes 1957 Passow et al 1961 Perkins 1961 Benesch & Benesch 1962) It seems reasonable however that dimethyl mercury with both valences of mercury bound to methyl groups in covalent non-dissociable bonds lacks this property Thus no chemical bonds to animal tissues are formed until dimethyl mercury is metabolised and one of the covalent bonds is split so that one of the valences of mercury is free to produce a dissociable com pound

The observation of a considerable amount of non volatile metabolites as early as 20 minutes after the administration indicates a fairly rapid metabolisation of dimethyl mercury in the body A high activity of the metabolites is seen in the liver and a metabolisation in the liver is not

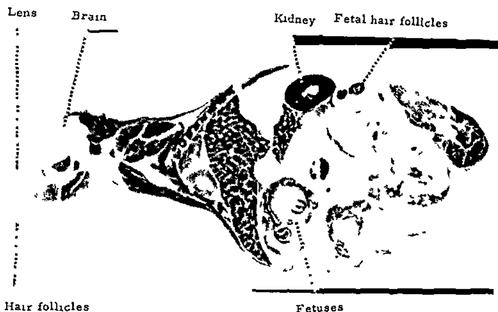


Fig 34 Autoradiogram representing non-volatile metabolites of ^{203}Hg dimethyl mercury 4 days after an i v injection of the compound in a pregnant mouse Note the fairly even distribution with the only tissue showing considerable accumulation being the kidney

surprising, since the liver is known to take part in many metabolisation processes The high activity of the metabolites in the bronchi of the lungs suggests that the metabolisation in part may take place in the bronchial epithelium This unexpected biochemical activity in the bronchi may demand a further comment

It is shown later in this work (Chapter XIV) that the metabolisation results in the formation of methyl mercury compounds and it is known from autoradiographic investigations (Berlin & Ullberg, 1963, Backstrom, 1969) that methyl mercury has no specific affinity to the bronchi Thus it seems as if the mercury has been transported to the bronchi in the dimethyl form and must consequently have been metabolised in the tissue where the metabolite was found This assumption is strongly supported by the findings of considerable concentrations of the metabolite in the bronchi of fetuses in pregnant mice This observation also shows that the uptake in the bronchi and the metabolisation occur independent of the respiration process

A considerable accumulation of the non-volatile compounds is also seen in the nasal mucosa and the mucous membranes of the oral part of the digestive system This is also the case in the fetuses in pregnant mice It seems plausible that a secretion of methyl mercury from the nasal mucosa occurs and that the labelled compound enters the oral cavity and is swallowed, causing the activity seen in the upper digestive tract It is known



Fig 35 Detail of fig 34 showing the distribution of activity in the fetus Note the high uptake in the lens of the eye

from autoradiographic investigations in mice (Backstrom 1969) that methyl mercury compounds accumulate in the nasal mucosa

A moderate activity of the metabolite was seen in some intestinal portions It may be explained by the swallowed activity in methyl mercury secreted from the nasal mucosa or by pollution of activity containing bile into the intestinal lumen It may also be due to an active secretion process of methyl mercury by the intestinal mucosa

Sixteen hours after administration no differences could be seen between the distribution pattern of dimethyl mercury plus metabolites as obtained from the autoradiograms of the surfaces of the deepfrozen blocks and the pattern of only non volatile metabolites This indicates that all dimethyl mercury is metabolised to non volatile compounds or has left the body by excretion routes i e that no radiomercury is present in the body as dimethyl mercury This elimination process must be completed later than 4 hours after administration and earlier than 16 hours after administration Thus all autoradiograms obtained after survival periods of 16 hours or more show distribution patterns only of the metabolite of dimethyl mercury

The distribution of this metabolite also very soon agreed in several respects with the distribution of intravenously injected methyl mercury in spite of the present localisation of the sites for formation of the metabolite The distribution pattern of the metabolite obtained after survival times of 24 hours and more shows no noticeable differences from the distribution patterns of methyl mercury compounds

The most obvious similarities in the behaviour at different post injection times between the metabolite and methyl mercury (according to the investigations by Berlin & Ullberg 1963 and Backstrom 1969) are the following

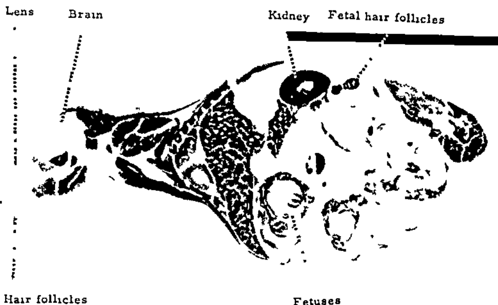


Fig 34 Autoradiogram representing non volatile metabolites of ^{203}Hg dimethyl mercury 4 days after an i v injection of the compound in a pregnant mouse Note the fairly even distribution with the only tissue showing considerable accumulation being the kidney

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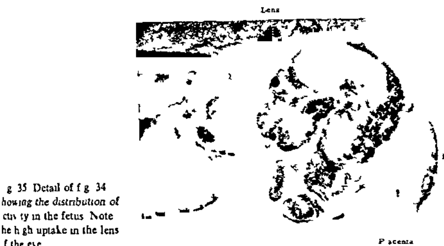


Fig 35 Detail of fig 34 *showing the distribution of activity in the fetus Note the high uptake in the lens of the eye*

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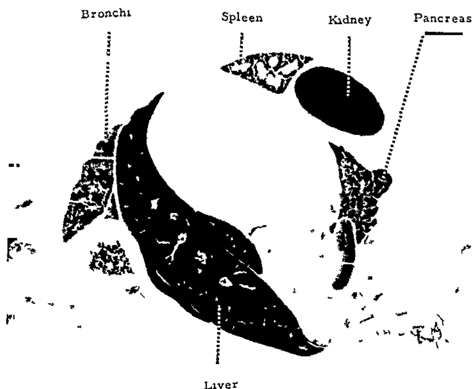


Fig 36 Detail of an autoradiogram showing only non volatile metabolites of dimethyl mercury 20 minutes after an i v injection of ^{203}Hg dimethyl mercury in a female mouse Note pronounced accumulation in the bronchi

The rapid uptake in the liver and the kidneys, in the adrenal cortex and in the nasal mucosa The behaviour in the digestive tract with an increase of the activity in the mucous membranes in the early stages The moderate and slow uptake in muscular tissue, the slowly increasing activity in the central nervous system and the slight accumulation in the hypophysis, the moderate uptake in the red pulp of the spleen, the uptake of activity in the lens of the eye and the very characteristic uptake in the fetal lens in pregnant mice The homogenous distribution picture after longer times of survival with the pronounced activity seen in the kidneys, the localization within the kidney and the activity observed in the basal parts of the hairs of the fur

All these characteristics and especially the high uptake in the lens of the fetal eye strongly suggest that the non-volatile metabolite of dimethyl mercury is the methyl mercury ion

Chapter XI. Retention of ^{203}Hg in mice after an intravenous injection of dimethylradiomercury studied by whole-body counting

The autoradiographic study (Chapter X) revealed a metabolisation process of dimethyl mercury in mice A rapid initial excretion of radiomercury followed by a slower excretion phase was indicated

The aim of this investigation was to elucidate the detailed retention course of ^{203}Hg after intravenous administration of ^{203}Hg labelled dimethyl mercury

Comments on materials and methods

Ten female mice were intravenously given 4 0—5 0 μCi ^{203}Hg labelled dimethyl mercury corresponding to the dose 12—15 mg Hg per kilo body weight

Immediately after the injection the mice were placed in the polyethylene container of the whole body measurement equipment and the measure ments were started The delay from the injection to the start of the measurements varied between 1 5 and 2.5 minutes

The whole body radioactivity was measured every two and a half minutes during the first half hour every fifth minute during the next half hour every fifteen minutes during the second hour every half hour during the third hour and thereafter in accordance with the indications in fig 37 and fig 38

The principles for the mathematical treatment of measurement data and the symbols used are presented in Chapter III (p 41 ff)

In order to get a value of the biological half life of the compound, responsible for the slow phase of excretion (vide infra) it was assumed that this compound was built in to the fur as was shown to be the case for methyl mercury (Chapter IV) Since the calculation of the retention function of the metabolite (valid for the body without skin and fur) by means of data processing should have demanded a specially designed data programme and since an exact $T_{1/2}$ value in this case was of limited value, an approximate estimation was made Thus the desired retention function was graphically estimated by hand and from this function the $T_{1/2}$ value was calculated In this estimation the time 2 days after administration was chosen as time zero

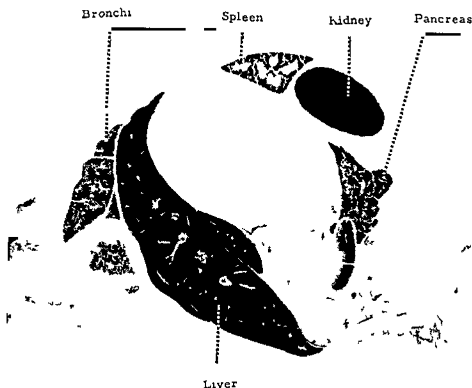


Fig 36 Detail of an autoradiogram showing only non-volatile metabolites of dimethyl mercury 20 minutes after an i v injection of ^{203}Hg dimethyl mercury in a female mouse Note pronounced accumulation in the bronchi

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All these characteristics and especially the high uptake in the lens of the fetal eye strongly suggest that the non volatile metabolite of dimethyl mercury is the methyl mercury ion

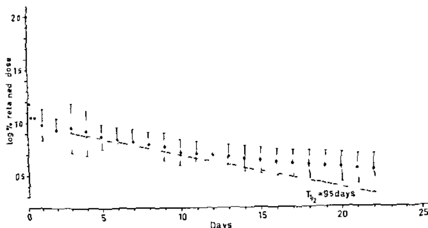


Fig 38 Retention of radiomercury during 22 days after an i v injection of dimethyl-radiomercury in mice Means of log % retained dose and 95 % confidence intervals for ten mice are given Dotted line represents estimated retention function valid for the body without skin and fur when the time two days after injection was chosen as time zero The biological half life for ^{203}Hg derived from this function is given

after administration of dimethyl mercury (Chapter XIII, fig 42) It may be concluded that the rapid excretion phase corresponded to the exhalation of dimethyl mercury

No attemps were made to analyse the exhalation course kinetically, since the essential information gained from the experiment was that a rapid excretion process in fact occurs, leaving only about 10 % of the initial radioactivity in the body and the detailed knowledge of the kinetics of this exhalation process seems to be of only secondary interest Of course the metabolised percentage of the dimethyl mercury should have been still lower than 10 % if the measurements could have started at the moment of injection in this case a certain part of the activity had already left the body when the whole body counting began

In the rest of the retention course, concerning the metabolite, there occurred individual variations, as seen from the width of the confidence intervals (fig 38) This may partly be explained by the variation between different mice in delay from injection of the compound to the starting of the measurements, which means that the time zero in one mouse was fixed later at the steep sloping initial part of the curve than in another mouse

The retention phase, corresponding to the excretion of the metabolite gave a retention function which fundamentally resembled the type of retention curve obtained for methyl mercury hydroxide (Chapter IV, Chapter V)

The dose of mercury given was in this experiment about 15 mg Hg per kilo body-weight. Two days after the administration (time zero for the estimation of the biological half-life of the metabolite) the retained per

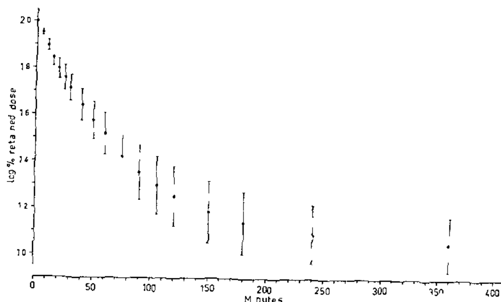


Fig 37 Retention of radiomercury during the first six hours after an i v injection of dimethylradiomercury in mice Means of log % retained dose and 95 % confidence intervals for ten mice are given

Results

The measurement data are presented in fig 37 and fig 38 as logarithmic percentage of the retained dose at each measuring time It will be seen that there was a rapid fall in the whole-body activity during the first hours after the injection, followed by a slow excretion phase for the remainder of the measurement period The rapid phase, as seen from fig 37, was completed after about six hours, leaving in mean ca 10 % of the initial radioactivity in the body The slow phase gave a retention curve with a slightly diminished decline towards the end of the measurement period (Chapter IV, Chapter V) The $T_{1/2}$-value for the metabolite calculated from the estimated retention function valid for the body without skin and fur was ≈ 9 5 days

Discussion

In the autoradiographic study (Chapter X) the mice killed four hours after injection seemed still to retain a small amount of mercury in the dimethyl form, while in the mice, killed after sixteen hours, no signs of dimethyl mercury were seen This observation agrees with the results of this investigation, showing that the rapid excretion phase was completed after about six hours From the graphic presentation of this rapid phase (fig 37) it will be seen that the type of function resembled the type of retention function obtained from the sampling of radioactivity in the exhaled air

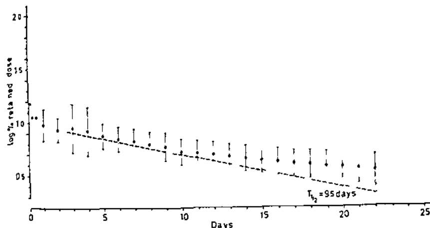


Fig 38 Retention of radiomercury during 22 days after an i v injection of dimethyl-radiomercury in mice Means of log % retained dose and 95 % confidence intervals for ten mice are given Dotted line represents estimated retention function valid for the body without skin and fur when the time two days after injection was chosen as time zero The biological half life for ^{203}Hg derived from this function is given

after administration of dimethyl mercury (Chapter XIII, fig 42) It may be concluded that the rapid excretion phase corresponded to the exhalation of dimethyl mercury

No attemps were made to analyse the exhalation course kinetically, since the essential information gained from the experiment was that a rapid excretion process in fact occurs, leaving only about 10 % of the initial radioactivity in the body and the detailed knowledge of the kinetics of this exhalation process seems to be of only secondary interest Of course the metabolised percentage of the dimethyl mercury should have been still lower than 10 % if the measurements could have started at the moment of injection, in this case a certain part of the activity had already left the body when the whole-body counting began

In the rest of the retention course, concerning the metabolite, there occurred individual variations, as seen from the width of the confidence intervals (fig 38) This may partly be explained by the variation between different mice in delay from injection of the compound to the starting of the measurements, which means that the time zero in one mouse was fixed later at the steep sloping initial part of the curve than in another mouse

The retention phase corresponding to the excretion of the metabolite gave a retention function which fundamentally resembled the type of retention curve obtained for methyl mercury hydroxide (Chapter IV, Chapter V)

The dose of mercury given was in this experiment about 15 mg Hg per kilo body-weight Two days after the administration (time zero for the estimation of the biological half life of the metabolite) the retained per-

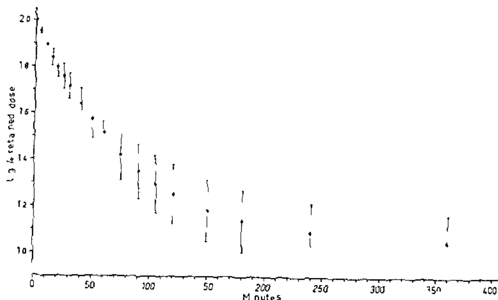


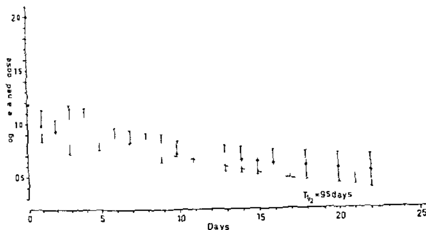
Fig 37 Retention of radiomercury during the first six hours after an i v injection of dimethylradiomercury in mice Means of log % retained dose and 95 % confidence intervals for ten mice are given

Results

The measurement data are presented in fig 37 and fig 38 as logarithmic percentage of the retained dose at each measuring time It will be seen that there was a rapid fall in the whole-body activity during the first hours after the injection followed by a slow excretion phase for the remainder of the measurement period The rapid phase, as seen from fig 37, was completed after about six hours, leaving in mean ca 10 % of the initial radioactivity in the body The slow phase gave a retention curve with a slightly diminished decline towards the end of the measurement period (Chapter IV Chapter V) The $T_{1/2}$ value for the metabolite calculated from the estimated retention function valid for the body without skin and fur was ≈9 5 days

Discussion

In the autoradiographic study (Chapter X) the mice killed four hours after injection seemed still to retain a small amount of mercury in the dimethyl form while in the mice killed after sixteen hours no signs of dimethyl mercury were seen This observation agrees with the results of this investigation showing that the rapid excretion phase was completed after about six hours From the graphic presentation of this rapid phase (fig 37) it will be seen that the type of function resembled the type of retention function obtained from the sampling of radioactivity in the exhaled air



F g 38 Retention of radiomercury during 22 days after an i v injection of dimethyl radiomercury in mice Means of log % retained dose and 95 % confidence intervals for ten mice are given Dotted line represents estimated retention function valid for the body without skin and fur when the time two days after injection was chosen as time zero The biological half life for ^{203}Hg derived from this function is given

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The retention phase corresponding to the excretion of the metabolite gave a retention function which fundamentally resembled the type of retention curve obtained for methyl mercury hydroxide (Chapter IV Chapter V)

The dose of mercury given was in this experiment about 15 mg Hg per kilo body weight Two days after the administration (time zero for the estimation of the biological half life of the metabolite) the retained per

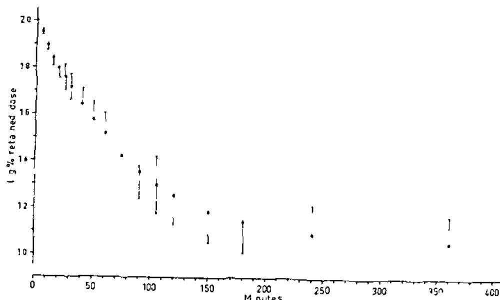


Fig 37 Retention of radiomercury during the first six hours after an i v injection of dimethylradiomercury in mice Means of log % retained dose and 95 % confidence intervals for ten mice are given

Results

The measurement data are presented in fig 37 and fig 38 as logarithmic percentage of the retained dose at each measuring time It will be seen that there was a rapid fall in the whole body activity during the first hours after the injection followed by a slow excretion phase for the remainder of the measurement period The rapid phase as seen from fig 37 was completed after about six hours leaving in mean ca 10 % of the initial radioactivity in the body The slow phase gave a retention curve with a slightly diminished decline towards the end of the measurement period (Chapter IV, Chapter V) The $T_{1/2}$ value for the metabolite calculated from the estimated retention function valid for the body without skin and fur was ~9 5 days

Discussion

In the autoradiographic study (Chapter X) the mice killed four hours after injection seemed still to retain a small amount of mercury in the dimethyl form while in the mice killed after sixteen hours no signs of dimethyl mercury were seen This observation agrees with the results of this investigation showing that the rapid excretion phase was completed after about six hours From the graphic presentation of this rapid phase (fig 37) it will be seen that the type of function resembled the type of retention function obtained from the sampling of radioactivity in the exhaled air

Chapter XII Retention of ^{203}Hg in mice after inhalation of dimethylradiomercury studied by whole-body counting

The aim of the investigation was to compare the retention of ^{203}Hg after inhalation of dimethylradiomercury with the retention obtained after intra venous injection (Chapter XI)

Comments on materials and methods

Ten female mice were given ^{203}Hg labelled dimethyl mercury by inhalation The activity given to each mouse varied between 1 5 and 3 0 μ Ci corresponding to the dose 4 5—9 0 mg Hg per kilo body weight

As soon as the mice had recovered from the ether anaesthesia (Chapter III p 30) they were placed in the whole body counting equipment and the measurements were started

The interval between the start of the inhalation and the start of the measurements varied approximately between 3 0 and 4 5 minutes

The whole body activity was measured at the same time after administration as in the preceding experiment The principles for the mathematical treatment of measurement data and the symbols used are presented in Chapter III (p 41 ff)

The retention function valid for the metabolite in the body without skin and fur was estimated as described in Chapter XI and from this function the value of biological half life of the metabolite was calculated

Results

The essentials of the measurement data are presented in fig 39 and fig 40 in the same manner as in the preceding experiment It will be seen from the figures that the retention course obtained in this experiment was very similar to the retention course obtained after an intravenous injection of dimethyl mercury (fig 37 and fig 38 Chapter XI) Also after inhalation of the compound there was a rapid excretion phase completed after about six hours, followed by a slow excretion course during the remaining part of the measurement period The retained amount of the given dose of radioactivity constituted in mean ca 19 % when the rapid phase was over The $T_{1/2}$-value for the metabolite calculated from the estimated retention function valid for the body without skin and fur was $\sim$7 days

centage of the dose was in mean 8 5 %, i e about 1 2 mg Hg per kilo body-weight The biological half-life of this dose of methyl mercury in mice should be about 8 9 days, as calculated by interpolation from the data obtained in Chapter V This value agrees fairly well with the value of biological half-life, obtained for the metabolite in this experiment, 9 5 days Thus the assumption that the non-volatile metabolite of dimethyl mercury in mice is monomethyl mercury (Chapter X and Chapter XIII) is supported

Chapter XII. Retention of ^{203}Hg in mice after inhalation of dimethylradiomercury studied by whole-body counting

The aim of the investigation was to compare the retention of ^{203}Hg after inhalation of dimethylradiomercury with the retention obtained after intravenous injection (Chapter XI).

Comments on materials and methods

Ten female mice were given ^{203}Hg labelled dimethyl mercury by inhalation. The activity given to each mouse varied between 1.5 and 3.0 μCi, corresponding to the dose 4.5—9.0 mg Hg per kilo body-weight.

As soon as the mice had recovered from the ether anaesthesia (Chapter III, p. 30) they were placed in the whole body counting equipment, and the measurements were started.

The interval between the start of the inhalation and the start of the measurements varied approximately between 3.0 and 4.5 minutes.

The whole body activity was measured at the same time after administration as in the preceding experiment. The principles for the mathematical treatment of measurement data and the symbols used are presented in Chapter III (p. 41 ff).

The retention function valid for the metabolite in the body without skin and fur was estimated as described in Chapter XI and from this function the value of biological half life of the metabolite was calculated.

Results

The essentials of the measurement data are presented in fig. 39 and fig. 40 in the same manner as in the preceding experiment. It will be seen from the figures that the retention course obtained in this experiment was very similar to the retention course obtained after an intravenous injection of dimethyl mercury (fig. 37 and fig. 38, Chapter XI). Also after inhalation of the compound there was a rapid excretion phase, completed after about six hours, followed by a slow excretion course during the [illegible] ining part [illegible] dose of [illegible] was over [illegible] retention [illegible] for the body without skin and fur was ~7 days.

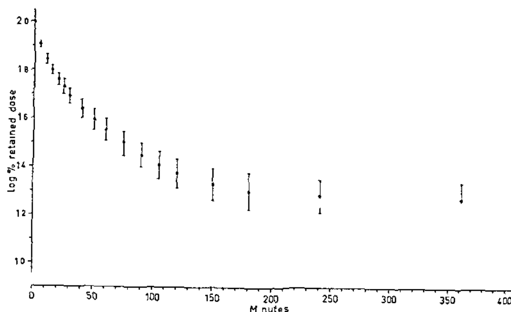


Fig 39 Retention of radiomercury during the first six hours after inhalation of dimethylradiomercury in mice Means of log % retained dose and 95 % confidence intervals for ten mice are given

Discussion

In Chapter X the assumptions were made that intact dimethyl mercury is inert towards animal tissues, that exhalation of the compound occurs as a result of its physical properties and that intravenous injection and inhalation are equivalent methods of administering dimethyl mercury as concerns the metabolic behaviour If these assumptions are correct, the retention course of ^{203}Hg after inhalation of dimethyl radiomercury should be identical to that after intravenous injection

The results of the present investigation showed that the main course of the obtained retention function was of the same two phase character as the retention curve obtained in the preceding investigation on mice given the compound by injection

The metabolised percentage of the administered dose was after 6 hours about 19 % when it was only about 10 % after intravenous injection This difference seems logical with regard to the longer elapse of the time between administration and the start of the measurements in this case, compared to the preceding investigation The influence of this interval on the obtained value for metabolised percentage of the dose is discussed in Chapter XI

The calculated dose of mercury retained in the body 2 days after the inhalation was in this case about 1 0 mg Hg per kilo body-weight. The biological half-life of this dose of methyl mercury should be ca 8 5 days as calculated from the data obtained in Chapter V

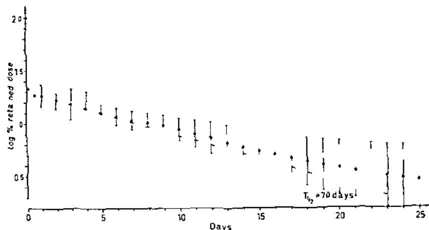


Fig 40 Retention of radiomercury during 25 days after inhalation of dimethyl radiomercury in mice Means of log % retained dose and 95 % confidence intervals for ten mice are given Dotted line represents estimated retention function valid for the body without skin and fur when the time two days after administration was chosen as time zero The biological half life for ^{203}Hg derived from this function is given

This value is of the same magnitude as the estimated value of the biological half life obtained for the metabolite in this experiment (about 7 days)

It may be concluded that the results of this experiment support the assumptions made that intravenous injection and inhalation are equivalent routes of administering dimethyl mercury as concerns the metabolic behaviour and that the physical properties of the intact compound are responsible for its interaction with animal tissues and consequently for the exhalation process

Chapter XIII. Quantification of exhaled radiomercury after intravenous injection of labelled dimethyl mercury

The aim of the investigation was to confirm the conclusions from the autoradiographic investigation and the whole body counting investigations on dimethyl mercury, which suggested that the rapid elimination of dimethyl mercury is due to an exhalation of radiomercury

Comments on materials and methods

Eight female mice were intravenously given ca 0 2 μCi of ^{203}Hg labelled dimethyl mercury, corresponding to a mercury amount of ca 1 3 μg

The injections were given when the mice were placed in the polyethylene container of the air sampling equipment (fig 6 p 34) Immediately after the injection the container was closed and the sampling started

The first collecting tube of the sampling equipment (tube A) was exchanged every five minutes during the first hour, every fifteen minutes during the second hour, every half hour during the third and fourth hours and then after five and six hours when the sampling was interrupted The other two collecting tubes (B and C) were exchanged after two hours and after six hours

Immediately after the end of the sampling period the mice were homogenized in 0 02 M aqueous $Hg(NO_3)_2$ solution using Ultraturrax TP 18/2 homogeniser

Two aliquots of the homogenate of each mouse were submitted to activity determination in gamma spectrometer together with the collected exhaled radiomercury

The dose of radioactivity given to each mouse in the form of labelled dimethyl mercury could not be exactly determined in advance because of the high volatility of the compound but had to be calculated from the experimental results Thus for each mouse the sum of the activity exhaled during the whole sampling period and the final total body activity as obtained from the homogenate was calculated and regarded as the total dose of radioactivity given

Results

All collected radioactivity was found in the first collecting tube of the sampling equipment (tube A) For each mouse the accumulated exhaled

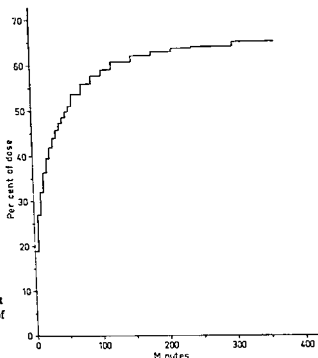


Fig 41 Accumulated excretion of radiomercury via the respiratory system during the first six hours after an i v injection of dimethylradiomercury The values represent the means of eight mice

amount of activity at the time for each exchange of collecting tube was calculated as per cent of the given dose The mean of these values are plotted against the time after the injection in fig 41 It will be seen that the exhalation of radioactivity was very rapid at the beginning of the experiment and then gradually decreased During the first five minutes after injection about 19 % of the given dose was exhaled, while the corresponding value for the fifth and sixth hours together was only about 1 %

The activity retained in the mouse at each time of exchange of collecting tube was calculated as per cent of the dose given These retention values at the different times after injection are plotted in fig 42 It will be seen that this retention curve is of the same type as the retention curves for dimethyl mercury, obtained by whole body counting (fig 37 and fig 39) The amount of radiomercury retained in the whole body six hours after the injection constituted in mean about 35 % of the given dose

Discussion

The exhalation rate of dimethyl mercury in mice was very high immediately after an intravenous injection and then gradually diminished until after six hours it was too slow to be measured by the method used These results confirm the assumption from the autoradiographic investigation

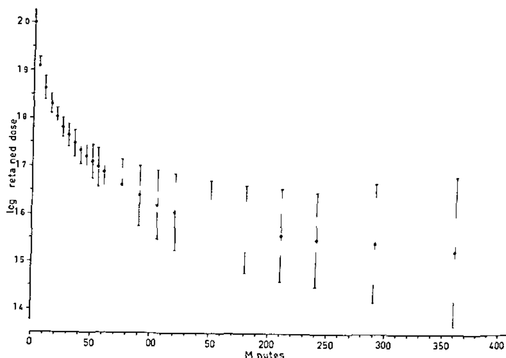


Fig 42 Retention of radiomercury after an i v injection of dimethylradiomercury as calculated from values for excretion through exhalation Means of eight mice and the 95 % confidence intervals are given

and the whole body counting experiments that the main part of the mercury after an intravenous injection of dimethyl mercury is exhaled

The type of retention curve obtained by calculation from exhalation values after inhalation of dimethyl radiomercury was similar to that obtained by whole body counting after intravenous injection or inhalation This observation supports the assumption that exhalation of radiomercury is responsible for the main excretion of mercury during the first six hours after administration of dimethyl mercury

In this investigation the mean value for the metabolised percentage of the dose after six hours was higher (about 35 %) than in the whole body counting investigations (10—20 %) The difference probably depends on the slow air flow rate used in this experiment (about 0 5 liter min $^{-1}$) compared to the flow rate used in the whole body counting experiments (about 4 liters min $^{-1}$) The slow rate necessary for the quantitative sampling of exhalation products probably caused a partial reinhalation of exhaled radiomercury resulting in a slower elimination process of dimethyl mercury from the animal It seems reasonable that the metabolisation rate of dimethyl mercury is in some way related to the concentration of dimethyl mercury in the metabolising tissues Consequently the metabolised part of the administered compound should be larger in this experiment than in the investigation using the whole body counting technique

The exhaled radioactivity was collected in the first tube of the sampling

equipment (tube A), containing a solution of $Hg(NO_3)_2$ No activity could be detected in the last tube (tube C), containing a solution of dithizone This indicates that the exhaled compound was intact dimethyl mercury, since most other organic mercury compounds or inorganic mercury should have passed the first tube and associated with the dithizone in the last collecting tube

Chapter XIV. Thin-layer chromatographic investigations on selected tissues and exhaled air after administration of dimethylradiomercury

The aim of the investigation was to study the chemical form in which radiomercury is present in the body and the form in which it is excreted via the respiratory system after administration of dimethylradiomercury

Comments on materials and methods

Five female mice were given about 40 μCi of ^{203}Hg-labelled dimethyl mercury by inhalation The amount of mercury given corresponded to about 20 mg Hg per kilo body-weight Immediately after the termination of the inhalation procedure two mice were placed in turn in the polyethylene container of the air sampling equipment, arranged for sampling of volatile compounds at $-78°$ C (p 35) Sampling was performed during the periods 0—10 minutes and 60—75 minutes, the starting of the sampling being time zero The ether solutions obtained were submitted to chromatography according to method B (p 22)

For examinations on the radiomercury in tissues one mouse was sacrificed five minutes after the inhalation procedure, one after one hour and one after 24 hours

From the mouse killed five minutes after the inhalation as much as possible of the retroperitoneal and the brown fat tissues were removed and homogenised and the volatile compounds were distilled off as described in Chapter III (p 37) The obtained ether solution was submitted to chromatography according to method B

From the mouse sacrificed after 1 hour, the kidneys were homogenised together with removed fat tissue Distillation was performed as described above and the remaining part of the homogenate was extracted according to Gage-Westoo (p 36) and the resulting benzene phase was submitted to chromatography according to method A (p 21)

From the mouse sacrificed 24 hours after the administration of dimethyl mercury, the liver and the kidneys were homogenised and extracted according to Gage-Westoo and chromatography was performed according to method A

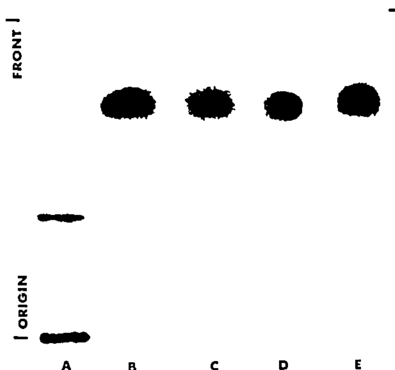


Fig 43 Radiochromatogram of tissue extract and sampled exhaled radiomercury after inhalation of ^{203}Hg labelled dimethyl mercury in mice A = φHgCN + φHgφ reference substances B = MeHgMe reference substance C and D Exhaled radiomercury sampled during the period 0—10 minutes and 60—75 minutes respectively after inhalation of $Me^{203}HgMe$ E – Extract of fat tissue from mouse sacrificed 5 minutes after administration of $Me^{203}HgMe$ Solvent system petroleum ether—diethyl ether (95 5) Temperature at development – 75° C

Results

Fig 43 shows the radiochromatogram obtained from the chromatography performed at low temperature in order to separate dimethyl mercury from other mercury compounds (method B) It will be seen that in the exhaled air from the two different periods after the administration only one labelled mercury compound could be detected and that this compound had the same R_f-value as dimethyl mercury As seen from the same chromatogram only one volatile labelled mercury compound was detected in the homogenate of fat tissue The R_f-value of this compound coincided with that of dimethyl mercury

The thin layer separation concerning non volatile organic radiomercury

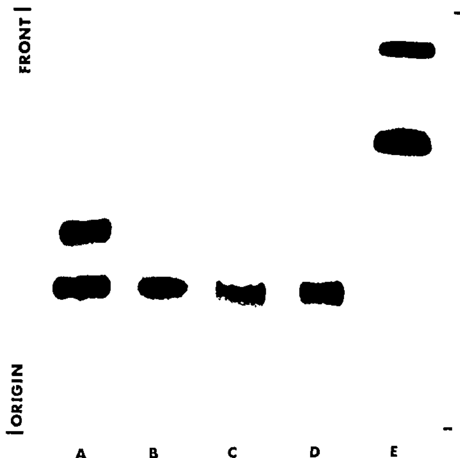


Fig 44 Radiochromatogram of tissue extracts from mice after inhalation of $Me^{203}HgMe$ A – MeHgCN + EtHgCN reference substances B = Extract of liver from mouse sacrificed 24 hours after administration of $Me^{203}HgMe$ C = Extract of kidney homogenate from which volatile compounds had been distilled off Time of survival 1 hour D = Extract of kidneys from mouse sacrificed 24 hours after inhalation of $Me^{203}HgMe$ E = φHgCN + φHgφ reference substances Solvent system diethyl ether toluene (90 10)

compounds in the tissue homogenates gave a chromatogram shown in fig 44 It revealed that only one single labelled mercury compound could be detected in the tissue homogenate from which volatile compounds had been distilled off and which was obtained from the mouse killed one hour after the inhalation of dimethyl mercury The R_f-value of this compound coincided with that of methyl mercury The chromatogram (fig 44) also showed in the homogenates of the liver and the kidneys of the mouse sacrificed 24 hours after inhalation of dimethyl mercury the occurrence of one single labelled mercury compound having the same R_f-value as methyl mercury

Discussion

It may be immediately concluded that the volatile compound exhaled was intact dimethyl mercury This conclusion agrees with the assumption made in Chapters X—XIII, that dimethyl mercury chemically and physically behaves as an inert substance toward animal tissues until it is metabolised to an ionisable compound This is also in accordance with the fact that dimethyl mercury was easily distilled off from tissue homogenates and chromatographically detected

The occurrence in tissues of a non volatile metabolite with the same R_f value as methyl mercury is in agreement with the findings from the autoradiographic investigation (Chapter X) and from the investigations on the retention function using whole body counting (Chapter XI and Chapter XII), indicating that dimethyl mercury is partly metabolised to methyl mercury This assumption may therefore be considered confirmed by the chromatographic results

It is of interest to note that in man, as pointed out by Swensson & Ulfvarson (1963) no definite differences have been seen between the symp toms of poisoning with dialkyl mercury compounds and the symptoms of poisoning with the corresponding alkyl mercury salts as they are described by Edwards (1865) Koelsch (1937, 1952), Hunter et al (1940) Hill (1943), Ahlmark (1948) Lundgren & Swensson (1949), Engleson & Herner (1952), and Hook et al (1954) The metabolic behaviour of dimethyl mercury in mice presented in this work, suggests that the described signs of poisoning after inhalation of dimethyl mercury in man may be caused by methyl mercury Of course, for the relevance of such an explanation it has to be revealed that the metabolic behaviour of dimethyl mercury in man is analogous to that in mice

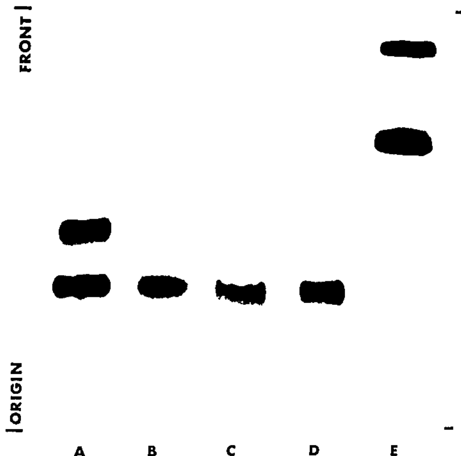


Fig 44 Radiochromatogram of tissue extracts from mice after inhalation of $Me^{203}HgMe$ A = MeHgCN + EtHgCN, reference substances B = Extract of liver from mouse, sacrificed 24 hours after administration of $Me^{203}HgMe$ C = Extract of kidney homogenate from which volatile compounds had been distilled off Time of survival 1 hour D = Extract of kidneys from mouse, sacrificed 24 hours after inhalation of $Me^{203}HgMe$ E = φHgCN + φHgφ reference substances Solvent system diethyl ether toluene (90 10)

compounds in the tissue homogenates gave a chromatogram, shown in fig 44 It revealed that only one single labelled mercury compound could be detected in the tissue homogenate, from which volatile compounds had been distilled off and which was obtained from the mouse killed one hour after the inhalation of dimethyl mercury The R_f-value of this compound coincided with that of methyl mercury The chromatogram (fig 44) also showed, in the homogenates of the liver and the kidneys of the mouse, sacrificed 24 hours after inhalation of dimethyl mercury, the occurrence of one single labelled mercury compound, having the same R_f-value as methyl mercury

It may be immediately concluded that the volatile compound exhaled was intact dimethyl mercury This conclusion agrees with the assumption made in Chapters X—XIII, that dimethyl mercury chemically and physically behaves as an inert substance toward animal tissues until it is metabolised to an ionisable compound This is also in accordance with the fact that dimethyl mercury was easily distilled off from tissue homogenates and chromatographically detected

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Summary and conclusions

In the introduction an outline is given of the essential features of the Swedish "mercury problem" which are of interest in the present investigation. The importance of obtaining detailed knowledge of the metabolic properties of the compounds involved, especially the methylated mercurials, is stressed. For more comprehensive descriptions of the mercury pollution as a problem of environmental hygiene, references to review works in the literature are given.

The aim of the present investigation was to study some metabolic properties of two organic mercury compounds, methyl mercury and dimethyl mercury, using radioactive substances. For this purpose, methods were developed for purification of commercially available, labelled methyl mercury to give a high degree of radiochemical purity. Dimethyl mercury was synthetised, labelled with ^{203}Hg, and purified. For synthesis of dimethyl mercury, a method described in the literature was modified to suit semi micro synthesis. Labelling of dimethyl mercury was performed by a simple exchange procedure. This method of labelling has not been found to be documented earlier for dialkyl or diaryl mercury compounds. For use as reference substances diphenyl mercury was synthetised, labelled (as described above) and purified, while the methoxyethyl mercury and ethyl mercury were labelled and purified. Commercially available phenyl mercury was purified according to an elaborated procedure (Chapter I).

Thin layer chromatographic methods were developed for separation and identification of mercury compounds (Chapter II). A combined adsorption partition chromatographic method is described for separation of organo mercurials of a moderate volatility. For identification of dimethyl mercury, the volatility of which is too large to permit separation by means of ordinary chromatographic procedures a method including development of thin layer plates at about $-75°$ C is described. For the determination of the ratio between inorganic, ionised mercury and organic mercury a method of separation of the substances in the form of dithizonates was used.

Chapter III reviews the general materials used and methods employed for the animal experiments. Inter alia equipment for sampling of exhaled labelled compounds metabolism cages for sampling of excreta and whole

body measurement equipment are described A method permitting extraction of about 97 per cent of methyl mercury from tissue homogenates is presented The applied mathematical treatment of measurement data is accounted for with a short description of programmes for data processing (vide infra)

In the animal experiments concerning the retention course of methyl mercury whole body radioactivity measurement technique was employed Methyl mercury is known to accumulate in hairs of different animals The mercury built in to the hairs is withdrawn from the circulation and may be regarded as excreted from the metabolic point of view That means that the whole body measurement comprises the sum of the mercury retained in the body and the amount of mercury transferred to and accumulated in the fur Since the mercury which has been accumulated in the hairs of the fur has no longer any significance for the metabolisation processes in the body it is of interest to know the relation between the amount of mercury in the body itself and in the fur for the evaluation of the whole body measurement data. An experiment, described in Chapter IV was performed in order to determine the retention function valid for the body without skin and fur The radioactivity retained in the body of animals without skin and fur was measured at different times after an injection of the labelled compound The total excretion of mercury per time unit after a single injection of methyl mercury was found to be approximately proportional to the simultaneous mercury concentration in the body at any given time This total excretion was found to occur mathematically as a reaction of the first order It is assumed that the components of this excretion function, i.e the transfer of mercury to the fur and the excretion via feces and urine also followed functions of the first order Then the retention function valid for radiomercury in the body without skin and fur could be obtained by calculation from the whole body measurement values of the mice From this function the biological half life of radiomercury as it is regulated by the sum of the excretion mechanisms could be derived ($T_{1/2}$) This procedure was applied on the data obtained in the whole body measurement investigations concerning the retention of methylradiomercury (Chapter V) The mathematical calculations were performed by data processing using the computer IBM 360/75 One programme, assisted by a subroutine was developed with the main purpose of calculating $T_{1/2}$ and one programme was developed for the calculation of data for graphic presentation of whole body measurement values

The whole body measurement investigations presented in Chapter V revealed that the biological half life of radiomercury after an intravenous injection is dependent on the total dose of stable methyl mercury given

The excretion rate was slower when larger doses were given The difference in biological half life between a trace dose and a large non toxic dose of methyl mercury was statistically highly significant.

Summary and conclusions

In the introduction, an outline is given of the essential features of the Swedish "mercury problem" which are of interest in the present investigation The importance of obtaining detailed knowledge of the metabolic properties of the compounds involved, especially the methylated mercurials, is stressed For more comprehensive descriptions of the mercury pollution as a problem of environmental hygiene, references to review works in the literature are given

The aim of the present investigation was to study some metabolic properties of two organic mercury compounds, methyl mercury and dimethyl mercury, using radioactive substances For this purpose methods were developed for purification of commercially available, labelled methyl mercury to give a high degree of radiochemical purity Dimethyl mercury was synthetised, labelled with ^{203}Hg, and purified For synthesis of dimethyl mercury, a method described in the literature was modified to suit semi micro synthesis Labelling of dimethyl mercury was performed by a simple exchange procedure This method of labelling has not been found to be documented earlier for dialkyl or diaryl mercury compounds For use as reference substances, diphenyl mercury was synthetised, labelled (as described above), and purified, while the methoxyethyl mercury and ethyl mercury were labelled and purified Commercially available phenyl mercury was purified according to an elaborated procedure (Chapter I)

Thin-layer chromatographic methods were developed for separation and identification of mercury compounds (Chapter II) A combined adsorption-partition chromatographic method is described for separation of organo mercurials of a moderate volatility For identification of dimethyl mercury, the volatility of which is too large to permit separation by means of ordinary chromatographic procedures a method including development of thin layer plates at about $-75°$ C is described For the determination of the ratio between inorganic ionised mercury and organic mercury a method of separation of the substances in the form of dithizonates was used

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The excretion rate was slower when larger doses were given The difference in biological half life between a trace dose and a large non toxic dose of methyl mercury was statistically highly significant

Two conceivable explanations of the slower excretion rate obtained at higher doses are discussed It may be due to an increasing mercurial inhibition of sulphydryl enzymes participating in excretion processes It may also be due to the binding of methyl mercury in the tissues to sites with different chemical properties Certain observations have suggested the occurrence of sites with different affinity (or availability) to methyl mer cury This also suggests that the mercury is not so easily released from sites with low availability (in the discussion called second class sites) as from sites with a high degree of availability (called first class sites) If that is the case large doses should be more slowly excreted than small doses since an increasing amount of second class sites will be occupied when larger doses are administered

No significant difference was seen between the sexes in the retention course of methyl mercury Nor was any significant change seen in the retention course of mice with a high degree of physical activity compared to control mice

A large dose of unlabelled methyl mercury, given six days before a trace dose of methylradiomercury, had only a slight influence on the radio mercury retention As an explanation of this phenomenon is proposed the suggested occurrence of tissue sites with different affinity (availability) to methyl mercury If the hypothesis is valid, the trace dose of radiomercury should be bound mainly to liberated first class sites and consequently be excreted fairly independently of the formerly given large dose of methyl mercury

In Chapter VI the distribution of methyl mercury between certain tissues was studied after administration of four different doses of stable methyl mercury hydroxide The aim of the investigation was not to study the distribution in particular but to elucidate if differences in distribution are seen when different doses of the compound are given The concentra tion of mercury in blood plasma was found to be approximately propor tional to the dose given and so was the concentration in the brain and in the kidneys Blood cells showed a statistically highly significant increase in uptake at higher doses while the uptake in muscular tissue and the liver showed a relatively decreased uptake (highly significant and significant respectively) when larger doses were given The results are discussed in relation to relevant literature and in relation to the hypothesis of occurrence of tissue sites of different affinity (availability) towards methyl mercury A potential difference between different tissues in the ratio *first class sites second class sites* could explain the observed changes in distribution pattern

Radioactivity measurements on exhalation products after injection of methylradiomercury (Chapter VII) revealed that if exhalation of radio mercury actually does occur it must be considered negligible Quantifica tion of radiomercury on sampled excreta showed that the main excretion

route is feces though a considerable *renal excretion* was seen, especially during the first days after administration

From extractions and thin layer chromatographic studies on tissue homogenates and excreta (Chapter VIII) *it is concluded* that no other organic mercury compounds are formed from methyl mercury in vivo It is also concluded that the dominant part of the mercury was present in the tissues as methyl mercury The main part of *excreted* mercury was found to be in the form of methyl mercury Pilot experiments (Chapter IX) suggested the occurrence of a break-down of a very small part of injected methyl mercury to inorganic mercury

In Chapter X the distribution of dimethylradiomercury was studied by whole body autoradiography after administration by intravenous injection and by inhalation The investigation was technically designed to give on one hand the distribution pattern of dimethyl mercury plus metabolites and on the other hand the pattern of only non-volatile metabolites

No differences in distribution were seen between mice, given dimethyl mercury by inhalation and mice, given the substance intravenously suggesting that the two ways of administration are equal The main part of the radioactivity left the body within a few hours, indicating the occurrence of an exhalation process

The principal features of the distribution of dimethyl mercury were the following

The concentration of dimethyl mercury decreased rapidly after administration in the circulating blood and the substance was simultaneously localised mainly to fat depots and to a lesser extent to tissues containing lipophilic cells The concentration in fetuses in pregnant mice was low *Four hours after administration the concentration of dimethyl mercury in* fat tissue was low and after sixteen hours no radiomercury was found to be present in the body in the form of dimethyl mercury

For non volatile metabolites the essential features of the distribution pattern were the following

During the first hours after administration an increasing radioactivity was seen in different structures, amongst which were the liver the kidneys, the adrenal cortex and the bronchi of the lungs In the fetuses of pregnant mice high activity was seen in the liver and in the bronchi of the lungs After longer times of survival the distribution pattern corresponding to only non volatile metabolites changed and showed after one day or more great similarities to the distribution pattern described in literature for methyl mercury The most striking detail in this similarity was the marked uptake in the fetal lens in pregnant mice It is suggested that intact dimethyl mercury behaves toward animal tissue as a chemically and physically inert substance It seems to be distributed in the body in accordance with its lipophilic character, and it is as a result of its physical properties, to a large extent exhaled It is partly metabolised to a non volatile metabolite,

the distribution pattern of which resembles that of methyl mercury The metabolisation was shown to occur, at least in part, in the bronchi of the lungs, independent of the process of respiration

In Chapter XI and Chapter XII, whole-body measurement studies were performed on the retention of radiomercury after administration of methyl-radiomercury by intravenous injection and by inhalation, respectively In both cases, the retention course could be divided into two parts, one rapid phase followed by a slow phase During the rapid phase, lasting for about six hours after the administration, 80—90 per cent of the administered radioactivity left the body It is assumed that this fast elimination was due to exhalation of radiomercury The slow phase, remaining for the rest of the measurement period (about three weeks) is discussed under the assumption that it corresponds to the excretion of the non volatile metabolite It had the same principal course as the retention course of methyl mercury, as this is described in Chapter V Biological half-lives of radio mercury, estimated from the slow excretion phase, showed fairly good agreement with biological half-lives of methyl mercury at corresponding dose levels It is concluded that the results of these experiments strengthen the assumptions that intravenous injection and inhalation are equal methods of administration of dimethyl mercury, as far as the metabolic behaviour is concerned, and that the metabolite is methyl mercury

Sampling and quantification of exhaled radiomercury (Chapter XIII) confirmed the assumption that the rapid excretion phase corresponds to exhalation of a radioactive compound Chromatographic investigations (Chapter XIV) revealed that the only detectable radioactive compound in exhaled air had the same R_f-value as dimethyl mercury In tissues of mice, killed at different times after administration of dimethylradiomercury, one volatile compound was detected, the R_f value of which coincided with that of dimethyl mercury In the same tissues a radiomercury compound of low volatility was detected This compound had the same R_f-value as methyl mercury

From these results together with the result of the preceding experiments using dimethyl mercury, it is concluded that the main part of dimethyl mercury administered to mice is rapidly exhaled The remaining part of the substance is metabolised to a non-volatile compound Dimethyl mercury seems to behave toward animal tissue as a chemically and physically inert substance until it is metabolised to an ionisable metabolite This metabolite was concluded to be methyl mercury

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References

Ahlmark, A Poisoning by methyl mercury compounds *Br J ind Med* 1948, **5**, 117—119

Akerfeldt, S Tiol- och disulfidgrupper i enzymer *Svensk kem Tidskr* 1964, **76**, 186—194

Backstrom J Distribution studies on mercuric pesticides in Japanese quail and some fresh water fishes *Acta pharmac tox* 1969 Suppl in press

Barron, E S G & G Kalnitsky The inhibition of succinoxidase by heavy metals and its reactivation with dithiols *Biochem J* 1947, **41**, 346—351

Benesch, R & R E Benesch Determination of -SH groups in proteins *Meth biochem Analysis* 1962, **10**, 43—70

Berg, W, A Johnels, B Lundholm, G Otterlind, P-I Persson, E Rosenberg, J Sondell, B Sjostrand, T Westermark, S Osterlof m fl Kvicksilver hos vattenlevande organismer *Kvicksilverfrågan i Sverige* Stockholm, 1965 a 25—33

Berg, W, A Johnels, E Rosenberg B Sjostrand, J Sondell, T Westermark & S Osterlof Kvicksilver och fiskgjuse *Kvicksilverfrågan i Sverige* Stockholm, 1965 b 69—76

Berg, W, A Johnels, B Sjostrand & T Westermark Mercury content in feathers of Swedish birds from the Past 100 years *Oikos* 1966, **17**, 71—83

Berglund, F Minamatasjukan *Lakartidn* 1967 **64**, 3531—3539

Berglund, F Toxicitetsexperiment med metylkvicksilverforeningar på råtta Nordiskt symposium kring kvicksilverproblematiken *Nord hyg Tidskr* 1969, **50** 118—124

Berlin, M Renal uptake, excretion and retention of mercury *Archs envir Hlth* 1963, **6**, 626—633

Berlin M & S Ullberg Accumulation and retention of mercury in the mouse I An autoradiographic study after a single intravenous injection of mercuric chloride II An autoradiographic comparison of phenylmercuric acetate with inorganic mercury III An autoradiographic comparison of methylmercuric dicyandiamide with inorganic mercury *Archs envir Hlth* 1963, **6** 589—616

Blatt, A H (Ed) Organic syntheses Wiley & Sons, London, 1948, vol II, 446

Boetius, J Lethal action of mercuric chloride and phenylmercuric acetate on fishes *Medd Danm Fiskeri- og Havsundersogelser* 1960 **3** 93—115

Borg, K Inverkan av betat utsade på viltfaunan Proc VIII Nord Veterinarmotet Helsingfors 1958 394—400

Borg K, H Wanntorp K Erne & E Hanko Alkyl mercury poisoning in the terrestrial Swedish wildlife *Viltrevy (Swedish wildlife)* 1969 **6** 301—379

Buckton G B Über die Isolierung des Quecksilbermethyls C_2H_3Hg *Justus Liebigs Annln Chem* 1858, **108**, 103—105

Buckton, G B Untersuchungen uber organische Metallverbindungen *Justus Liebigs Annln Chem* 1859, **109**, 218—227

Buckton, G B On the formation of organo-metallic radicles by substitution *Trans Chem Soc* 1863, 16 18—25
Boyer, P D Sulfhydryl and disulfide groups of enzymes In Boyer, P D, H Lardy & K Myrback (Eds) The enzymes, Vol 1, 511—588 Acad Press Inc New York, 1959
Clarkson, T & A Rothstein The excretion of volatile mercury by rats injected with mercury salts *Hlth Phys* 1964, 10, 1115—1121
Clarkson, T W, A Rothstein & R Sutherland The mechanism of action of mercurial diuretics in rats, The metabolism of 203Hg labelled chlormerodrin *Br J Pharmac Chemother* 1965, 24, 1—13
Cohen E N & N Hood Application of low-temperature autoradiography to studies of the uptake and metabolism of volatile anesthetics in the mouse *Anesthesiology* 1969, 30, 306—314
Dinman B D E E Evans & A L Linch Organic mercury Environmental exposure, excretion and prevention of intoxication in its manufacture *A M A Archs ind Hlth* 1958 18, 248—260
Dixon, W J & F J Massey Introduction to statistical analysis McGraw Hill Book Company, Inc London, 1951
Dreher, E & R Otto Über Quecksüberdiphenyl *Justus Liebigs Annln Chem* 1870, 154, 93—130
Edvardson, K, L Ekman & U Greitz An attempt to application of a multi compartment model Rep Res Inst National Defence, Dept 4, Stockholm, Sweden, 1964 A 4391—4623 1—15
Edwards G N Two cases of poisoning by mercuric methide *St Bart s Hosp Rep* 1865 1, 141—150
Ekman, L Distribution and excretion of radio-cesium in goats pigs and hens *Acta vet scand* 1961, 2 suppl 4
Ekman, L U Greitz, G Persson, & B Åberg Omsattning av metylkvicksilver hos manniska *Nord Med* 1968 79, 450—456
Engleson G & T Herner Alkyl mercury poisoning Acta paediat Stockh 1952 41 289—294
Eriksson, K H Studies on the metabolism of mercury in mice FORTRAN programme for mathematical treatment and statistical description of whole body measurement data Rep Res Inst National Defence, Dept 1, Stockholm, Sweden, 1969 *In press*
Fildes P The mechanism of the anti bacterial action of mercury *Brit J Exper Path* 1940 21 67—73
Fischer R A Statistical methods for research workers Oliver and Boyd London, 1950
Fischer R A & F Yates Statistical tables for biological agricultural and medical research Oliver and Boyd London, 1949
Frankland E Einwirkung von Quecksilber auf Jodmethyl in Licht *Justus Liebigs Annln Chem* 1853 85, 361—364
Frankland E & B F Duppa On a new method of producing the mercury compounds of the alcohol radicals *Transactions of the Chemical Society* 1863, 16, 415—425
Freyschuss S O Lindstrom K D Lundgren & A Swensson Yrkeshygieniska undersokningar i samband med anvandande av organiska kvicksilverforeningar inom pappersbruk och exportslipen *Svensk Papp Tidn* 1958, 61, 568—580
Friberg L Studies on the metabolism of mercuric chloride and methyl mercury dicyandiamide *AMA Archs ind Hlth* 1959, 20, 42—49
Friberg L E Odeblad & S Forssman Distribution of two mercury compounds in rabbits after a single subcutaneous injection *AMA Archs ind Hlth* 1957, 16,

References

Ahlmark, A Poisoning by methyl mercury compounds *Br J ind Med* 1948, **5**, 117—119

Åkerfeldt, S Tiol- och disulfidgrupper i enzymer *Svensk kem Tidskr* 1964, **76**, 186—194

Backstrom, J Distribution studies on mercuric pesticides in Japanese quail and some fresh water fishes *Acta pharmac tox* 1969 Suppl in press

Barron, E S G & G Kalnitsky The inhibition of succinoxidase by heavy metals and its reactivation with dithiols *Biochem J* 1947, **41**, 346—351

Benesch, R & R E Benesch Determination of -SH groups in proteins *Meth biochem Analysis* 1962, **10**, 43—70

Berg W, A Johnels, B Lundholm, G Otterlind, P-I Persson, E Rosenberg, J Sondell, B Sjostrand, T Westermark, S Österlof m fl Kvicksilver hos vattenlevande organismer *Kvicksilverfrågan i Sverige* Stockholm, 1965 a, 25—33

Berg, W, A Johnels, E Rosenberg, B Sjostrand, J Sondell, T Westermark & S Österlof Kvicksilver och fiskgjuse *Kvicksilverfrågan i Sverige* Stockholm, 1965 b, 69—76

Berg, W, A Johnels, B Sjostrand & T Westermark Mercury content in feathers of Swedish birds from the Past 100 years *Oikos* 1966, **17**, 71—83

Berglund, F Minamatasjukan *Lakartidn* 1967 **64** 3531—3539

Berglund, F Toxicitetsexperiment med metylkvicksilverforeningar på råtta Nordiskt symposium kring kvicksilverproblematiken *Nord hyg Tidskr* 1969, **50** 118—124

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Blatt, A H (Ed) Organic syntheses Wiley & Sons, London 1948 vol II, 446

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Borg, K Inverkan av betat utsade på viltfaunan Proc VIII Nord Veterinärmotet Helsingfors, 1958 394—400

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Buckton G B Über die Isolierung des Quecksilbermethyls C_2H_3Hg *Justus Liebigs Annln Chem* 1858, **108**, 103—105

Buckton, G B Untersuchungen über organische Metallverbindungen *Justus Liebigs Annln Chem* 1859, **109**, 218—227

Ladenburg, A Über neue Siliciumberbindungen *Justus Liebigs Annln Chem* 1874, 173, 143—166
Landell, N E Fageldod fiskbot kvicksilver Aldus/Bonniers, Stockholm, 1968
Laug E P & F M Kunze The absorption of phenylmercuric acetate from the vaginal tract of the rat *J Pharmac exp Ther* 1949, 95, 460—464
Lindstrom, O Liquid seed treatment studies. *Trans Royal Inst Technol* Stockholm, 1961, 185
Lundgren, K D & A Swensson Occupational poisoning by alkyl mercury compounds *J ind Hyg Toxicol* 1949, 31, 190—200
Maren, T H A simple and accurate method for the determination of mercury in biologic material *J Lab clin Med* 1943, 28, 1511—1514
Maynard J L The direct mercuration of benzene and the preparation of mercury diphenyl *J Am chem Soc* 1924 46, 1510—1512
Miettinen, J K, T Rahola T Hattula, K Rissanen & M Tillander Retention and excretion of 203Hg-labelled methyl mercury in man after oral administration of CH_3 203Hg biologically incorporated into fish muscle protein Paper presented at Fifth symposium on radioactivity in Scandinavia, Helsinki, May, 19—20, 1969 a
Miettinen, V, Y Ohmomo M Valtonen, E Blankenstein, K Rissanen, M Tillander & J K Miettinen Preliminary notes on the distribution and effects of two chemical forms of methyl mercury on pike Paper presented at Fifth symposium on radioactivity in Scandinavia, Helsinki, May, 19—20, 1969 b
Miller, V L, G E Bearse, Y Kimura & E Csonka Strain differences in mercury retention by chicks *Proc Soc exp Biol Med* 1959 a, 100, 301—303
Miller, V L, G E Bearse & K E Hammermeister Mercury retention a trait of chickens *Poult sci* 1959 b, 38, 1037—1039
Miller, V L & E Csonka Mercury retention in two strains of mice *Toxic appl Pharmac* 1968, 13, 207—211
Miller, V L, V Larkin, G E Bearse & C M Hamilton The effects of dosage and administration of two mercurials on mercury retention in two strains of chickens *Poult Sci* 1967 64 142—146
Moriyama H A study on the congenital Minamata Disease *Kumamoto Igk Z* 1968, 41 506—532 (In Japanese)
Nordiskt symposium kring kvicksilverproblematiken, Lidingo, October, 10—11, 1968 *Nord hyg Tidskr* 1969, 50
Norén K & G Westoo Metylkvicksilver i fisk *Vår foda* 1967, 19, 13—17
Ohmomo Y V Miettinen E Blankenstein & J K Miettinen Studies on the distribution of 203Hg labelled methyl mercury and phenyl mercury in pike Paper presented at Fifth symposium on radioactivity in Scandinavia, Helsinki, May, 19—20 1969
Passow H A Rothstein & T W Clarkson The general pharmacology of the heavy metals *Pharmac Rev* 1961, 13 185—224
Paulsson S & B Åberg Kliniskt kemiska och hematologiska normalvarden *Svensk Vet Tidn* 1965 15, 462—476
Perkins D J A study of the amino-acid complexes formed by metals of group II of the periodic classification *Biochem J* 1952 51, 487—490
Perkins D J A study of the effect of amino acid structure on the stabilities of the complexes formed with metals of group II of the periodic classification *Biochem J* 1953 55 649—653
Perkins D J Studies on the interaction of zinc, cadmium and mercuric ions with native and chemically modified human serum albumin *Biochem J* 1961, 80, 668—672.
[illegible]ope R S Collection of mouse urine or bile *J Physiol* 1967, 22, 1024—1025
[illegible]aeder, M G & E Snekvik Quecksilberhalt mariner Organismen. *Kongl Vidensk Selsk Forhandl* 1941, 13, 169—172.

Gage J C The trace determination of phenyl and methylmercury salts in biological material *Analyst* Lond 1961 **86** 457—459

Gage J C Distribution and excretion of methyl and phenyl mercury salts *Br J ind Med* 1964 **21**, 197—202

Gage J C & A B Swan The toxicity of alkyl and aryl mercury salts *Abstr Biochem Pharmac* 1961 **8** 77

Hagen U Toxikologie organischer Quecksilberverbindungen *Naunyn Schmiedebergs Arch exp Path Pharmak* 1955 **224** 193—205

Hannerz L Experimental investigations on the accumulation of mercury in water organisms *Rep Inst Freshw Res Stockholm* Sweden 1968 **48** 120—176

Hein F & K Wagler Eine neue Methode zur Darstellung symmetrischer Organoquecksilberverbindungen *Ber dt chem Ges* 1925 **58** 1499—1509

Hill W H A report on two deaths from exposure to the fumes of a di ethyl mercury *Can J publ Hlth* 1943 **34** 158—160

Hook O K D Lundgren & A Swensson On alkyl mercury poisoning with a description of two cases *Acta med scand* 1954 **150** 131—137

Hoshino O K Tanzawa T Terao & T Ukita Quantitative determination of mercury in hair by activation analysis *J hyg chem Soc Japan* 1966 **12** 94—99

Hughes W L A physicochemical rationale for the biological activity of mercury and its compounds *Ann N Y Acad Sci* 1957 **65** 454—460

Hughes W L R Straessle H Edelhoch & J T Edsall Equilibrium kinetics and formation of intermediates in the reaction of human mercaptalbumin with mercury compounds Abstract of papers the div of Biol Chem Am Chem Soc 1950 51 C—52 C

Hunter D R R Bromford & D S Russel Poisoning by methyl mercury compounds *Q Jl Med* 1940 **33** 193—213

Informationskonferens rorande kvicksilver *Kvicksilverfragan i Sverige* Stockholm September 8 1965

Jacobsson S O & P Lindberg The influence of a flushing dose of selenium on the excretion of selenium in sheep *Acta vet scand* 1968 **9** 199—207

Jensen S & A Jernelov Biosyntes av metylkvicksilver I Slam *Biocidinformation* 1967 **10** 4—5

Jensen S & A Jernelov Biosyntes av metylkvicksilver II Bildning av dimetyl kvicksilver vid forruttnelse av fisk *Biocidinformation* 1968 **14** 3—5

Jervis R E A K Perkins W D McIntosh & M F Kerr Activation analysis in forensic investigation Proc 1961 intern conf modern trends in activation analysis College station Texas 1962 107—113

Johnels A G M Olsson & T Westermark Kvicksilver i fisk *Var foda* 1967 **7** 68—103

Johnels A G M Olsson & T Westermark Esox lucius and some other organisms as indicators of mercury contamination in Swedish lakes and rivers *Bull Off int Epizoot* 1968 **69** 1439—1452

Kekulé A & A Franchimont Über das Triphenylmethan *Ber dt chem Ges* 1872 **5** 906—908

Kimura Y & V L Miller The degradation of organo mercury fungicides in soil *J agric Fd Chem* 1964 **12** 253—257

Koelsch F Gesundheitsschadigungen durch organische Quecksilberverbindungen *Arch Gewerbepath Gewerbehyg* 1937 **8** 113—116

Koelsch F Vergiftungen durch organische Quecksilberverbindungen *Atti Congresso Intern Med del Lavoro* Milano 1952 103—106

Kurland I T Faro N Stanley & H Siedler Minamata Disease *Wld Neur I* 1960 **1** 370—395

Ladenburg A Über neue Siliciumverbindungen *Justus Liebigs Annln Chem* 1874 173 143—166
Landell N E Fågeldod fiskhot kvicksilver *Aldus/Bonniers* Stockholm 1968
Laug, E P & F M Kunze The absorption of phenylmercuric acetate from the vaginal tract of the rat *J Pharmac exp Ther* 1949 95 460—464
Lindstrom O Liquid seed treatment studies *Trans Royal Inst Technol* Stockholm 1961 185
Lundgren K D & A Swensson Occupational poisoning by alkyl mercury compounds *J ind Hyg Tox col* 1949 31 190—200
Maren, T H A simple and accurate method for the determination of mercury in biologic material *J Lab clin Med* 1943 28 1511—1514
Maynard, J L The direct mercuration of benzene and the preparation of mercury diphenyl *J Am chem Soc* 1924 46 1510—1512
Miettinen J K T Rahola T Hattula K Rissanen & M Tillander Retention and excretion of ^{203}Hg labelled methyl mercury in man after oral administration of CH_3 ^{203}Hg biologically incorporated into fish muscle protein. Paper presented at Fifth symposium on radioactivity in Scandinavia Helsinki May, 19—20 1969 a
Miettinen V., Y Ohmomo M Valtonen, E. Blankenstein K. Rissanen M Tillander & J K Miettinen Preliminary notes on the distribution and effects of two chemical forms of methyl mercury on pike Paper presented at Fifth symposium on radioactivity in Scandinavia Helsinki May 19—20 1969 b
Miller V L G E Bearse, Y Kimura & E Csonka Strain differences in mercury retention by chicks *Proc Soc exp Biol Med* 1959 a 100 301—303
Miller V L G E Bearse & K E Hammermeister Mercury retention a trait of chickens *Poult sci* 1959 b 38 1037—1039
Miller V L & E Csonka Mercury retention in two strains of mice *Toxic appl Pharmac* 1968 13 207—211
Miller V L V Larkin G E Bearse & C M Hamilton The effects of dosage and administration of two mercurials on mercury retention in two strains of chickens *Poult Sci* 1967 64 142—146
Moriyama H A study on the congenital Minamata Disease *Kumamoto Igk Z* 1968 41 506—532 (In Japanese)
Nordiskt symposium kring kvicksilverproblematiken Lidingö October 10—11 1968 *Nord hyg Tidskr* 1969 50
Norén K & G Westoo Metylkvicksilver i fisk *Var foda* 1967 19 13—17
Ohmomo Y V Miettinen E Blankenstein & J K Miettinen Studies on the distribution of ^{203}Hg labelled methyl mercury and phenyl mercury in pike Paper presented at Fifth symposium on radioactivity in Scandinavia, Helsinki May 19—20, 1969
Passow H A Rothstein & T W Clarkson The general pharmacology of the heavy metals *Pharmac Rev* 1961 13 185—224
Paulsson S & B Åberg Kliniskt kemiska och hematologiska normalvarden *Svensk Vet Tidn* 1965 15 462—476
Perkins D J A study of the amino-acid complexes formed by metals of group II of the periodic classification *Biochem J* 1952 51 487—490
Perkins D J A study of the effect of amino acid structure on the stabilities of the complexes formed with metals of group II of the periodic classification *Biochem J* 1953 55 649—653
Perkins, D J Studies on the interaction of zinc cadmium and mercuric ions with native and chemically modified human serum albumin *Biochem J* 1961 80 668—672
Pope R S Collection of mouse urine or bile *J Physiol* 1967 22 1024—1025
Raeder M G & E. Snekvik Quecksilberhalt mariner Organismen *Kongl Vidensk Selsk Forhandl* 1941 13 169—172

Reitman, S & S Frankel A colorimetric method for the determination of serum glutamic oxalacetic and glutamic puruvic transaminases *Am J clin Path* 1957, **28**, 56—63

Riehm, E *Zentr Bakteriol Parasitenk* 1914, **40**, 424 Cit Ulfvarson, 1961

Schroeder, H Dichtigkeitsmessungen fester organischer Verbindungen *Ber dt chem Ges* 1879, **12**, 561—567

Seidel M Über die Oxydation des Quecksilberdiathyls mit ubermangansaurem Kali *J prakt Chem* 1884, **29**, 134—136

Spencer, H, M Roque, M Li & I Samachson The effect of stable strontium on radiostrontium metabolism in man *Fedn Proc Fedn Am Socs exp Biol* 1961, *20, 290*

Spieckermann, A *Ill Landwirtsch Ztg* 1914, **34**, 665 Cit Ulfvarson, 1961

Sporek, K F Determination of mercury in fungicidal preparations containing organo mercury compounds Part II The determination of organo-mercury compounds after decomposition to mercuric sulphide *Analyst*, Lond 1956,**81**, 478—482

Stock A Die mikroanalytische Bestimmung des Quecksilbers und ihre Anwendung auf hygienische und medizinische Fragen *Svensk Kem Tidskr* 1938 a, **50**, 242—250

Stock, A Der Quecksilbergehalt des Menschlichen Organismus *Biochem Z* 1940, **304**, 73—80

Stock, A Zur mikroanalytischen Bestimmung des Quecksilbers *Mikrochemie mikrochem Acta* 1942, **30**, 128—136

Stock, A & N Neuenschwander Lemmer Die mikroanalytische Quecksilberbestimmung (XXVII Mitteil uber Wirkung und Verbreitung des Quecksilbers) *Ber dt chem Ges* 1938 b,**71**: **1** 550—562

Sumino, K Analysis of organic mercury compounds by gas chromatography *Kobe J Med Sci* 1968 **14**, 131—148

Suzuki T T Miyama & H Katsunuma A comparative study on the distribution and excretion of several mercuric compounds in rat *Jap J med Prog* 1961 **48**, 716—722

Suzuki, T, T Miyama & H Katsunuma Comparative study of bodily distribution of mercury in mice after subcutaneous administration of methyl, ethyl and n-propyl mercury acetates *Jap J exp Med* 1963 **33**, 277—282

Swensson Å Investigations on the toxicity of some organic mercury compounds which are used as seed disinfectants *Acta med scand 1952*, **143** *365—384*

Swensson Å, K-D Lundgren & O Lindstrom Distribution and excretion of mercury compounds after single injection *A M A Archs ind Hlth* 1959 a **20** 432—443

Swensson Å K-D Lundgren & O Lindstrom Retention of various mercury compounds after subacute administration *A M A Archs ind Hlth* 1959 b **20** 467—472

Swensson Å & U Ulfvarson Toxicology of organic mercury compounds used as fungicides *Occup Hlth Rev* 1963 5—11

Swensson Å & U Ulfvarson Experiments with different antidotes in acute poisoning by different mercury compounds Effects on survival and on distribution and excretion of mercury *Int Arch Gewerbepath Gewerbehyg* 1967 **24** 12—15

Swensson, Å & U Ulfvarson Distribution and excretion of various mercury compounds after single injections in poultry *Acta pharmac tox* 1968 a **26** 259—272

Swensson Å & U Ulfvarson Distribution and excretion of mercury compounds in rats over a long period after a single injection *Acta pharmac tox* 1968 b **26** 273—283

Takeda Y T Kunugi O Hoshino & T Ukita Distribution of inorganic aryl and alkyl mercury compounds in rats *Toxic appl Pharmac* 1968 a 13 156—164

Takeda Y, T Kunugi T Terao & T Ukita Mercury compounds in the blood of rats treated with ethyl mercuric chloride *Toxic appl Pharmac* 1968 b 13 165—173

Takeuchi T N Morikawa H Matsumoto & Y Shiraishi A pathological study of Minamata Disease in Japan *Acta Neuropath* 1962 2 40—57

Tejning S Biological effects of methyl mercury dicyandiamide treated grain in the domestic fowl Gallus gallus L VI Quantitative studies on the fate of ingested mercury Elimination in eggs and excrement deposition in the body and distribution in organs *Oikos* 1967 a Suppl 8 45—51

Tejning S Biological effects of methyl mercury dicyandiamide treated grain in the domestic fowl Gallus gallus L VII Alimentary methyl mercury poisoning in a cock—food consumption clinical signs and deposition of mercury in organs *Oikos* 1967 b Suppl 8 52—55

Tejning S Biological effects of methyl mercury dicyandiamide treated grain in the domestic fowl Gallus gallus L X Mercury in organs in new hatched chicks *Oikos* 1967 c Suppl 8 67 74

Tejning S Biological effects of methyl mercury dicyandiamide treated grain in the domestic fowl Gallus gallus L XI Juvenile development general health and mercury in organs in chicks hatched from eggs with presumed mercury contents of 0 2—0 4 mg *Oikos* 1967 d Suppl 8 75—76

Tejning S Biological effects of methyl mercury dicyandiamide-treated grain in the domestic fowl Gallus gallus L XII Effects on food consumption and egg laying symptomatology deposition of mercury in blood and organs elimination of mercury in excrement and eggs and transportation to the plumage *Oikos* 1967 e Suppl 8 77—110

Tejning S Kvicksilverhalten i blodkroppar och i blodplasma hos arbetare exponerade for metylkvicksilverforening vid betning av utsade Rep no 67 02 10 from Yrkesmedicinska Kliniken Lasarettet Lund Sweden Lund 1968 a

Tejning S Kvicksilverhalterna i blodkroppar blodplasma och har hos storkonsumenter av fisk fran olika omraden av Vanern och sambandet mellan kvicksilverhalterna i dessa organelement och kvicksilverhalten i fisk samt forslag om det internationella fodoameshygieniska gransvardets for kvicksilver tillampning pa fisk och fiskprodukter Rep no 67 08 31 from Yrkesmedicinska Kliniken Lasarettet Lund Sweden Lund 1968 b

Tejning S & R Westerberg Alkyl mercury treated seed in food grain Mercury in tissues and eggs from hens fed with grain containing methyl mercury dicyandiamide *Poult Sci* 1964 43 6—11

The mercury problem Symposium concerning mercury in the environment Stockholm Januari 24—26 1966 *Oikos* 1967 Suppl 9

Tillander M Studies on excretion rates of organic mercury compounds in seal and fish Paper presented at Fifth symposium on radioactivity in Scandinavia Helsinki May 19—20 1969

Torgeson D C Fungicides Vol I Acad Press Inc New York 1967

Tonbara T Y & C P Shields The analysis of submicrogram amounts of mercury in tissues *Am ind Hyg Ass J* 1968 29 87—93

Ukita T Y Takeda, Y Sato & T Takahashi Distribution of ^{203}Hg labelled mercury compounds in adult and pregnant mice determined by whole body autoradiography *Radio-Isotopes* Tokyo 1967 16 439—448 (In Japanese)

Ulfvarson U Betning av utsade *Svensk Kem Tidskr* 1961 73 553—565

Ulfvarson U Distribution and excretion of some mercury compounds after long term exposure *Int Arch Gewerbepath Gewerbehyg* 1962 19 412—422

Reitman, S & S Frankel A colorimetric method for the determination of serum glutamic oxalacetic and glutamic puruvic transaminases *Am J clin Path* 1957, **28**, 56—63

Riehm, E *Zentr Bakteriol Parasitenk* 1914, **40**, 424 Cit Ulfvarson, 1961

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Spencer, H, M Roque, M Li & I Samachson The effect of stable strontium on radiostrontium metabolism in man *Fedn Proc Fedn Am Socs exp Biol* 1961, **20**, 290

Spieckermann, A *Ill Landwirtsch Ztg* 1914, **34**, 665 Cit Ulfvarson, 1961

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Stock, A Der Quecksilbergehalt des Menschlichen Organismus *Biochem Z* 1940, **304**, 73—80

Stock, A Zur mikroanalytischen Bestimmung des Quecksilbers *Mikrochemie mikrochem Acta* 1942, **30**, 128—136

Stock, A & N Neuenschwander-Lemmer Die mikroanalytische Quecksilberbestimmung (XXVII Mitteil uber Wirkung und Verbreitung des Quecksilbers) *Ber dt chem Ges* 1938 b **71: 1** 550—562

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Suzuki, T, T Miyama & H Katsunuma A comparative study on the distribution and excretion of several mercuric compounds in rat *Jap J med Prog* 1961 **48** 716—722

Suzuki T, T Miyama & H Katsunuma Comparative study of bodily distribution of mercury in mice after subcutaneous administration of methyl ethyl and n propyl mercury acetates *Jap J exp Med* 1963, **33** 277—282

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Swensson, Å, K-D Lundgren & O Lindstrom Distribution and excretion of mer cury compounds after single injection *A M A Archs ind Hlth* 1959 a **20** 432—443

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Swensson Å & U Ulfvarson Toxicology of organic mercury compounds used as fungicides *Occup Hlth Rev* 1963, 5—11

Swensson Å & U Ulfvarson Experiments with different antidotes in acute poisoning by different mercury compounds Effects on survival and on distribution and excretion of mercury *Int Arch Gewerbepath Gewerbehyg* 1967 **24** 12—15

Swensson, Å & U Ulfvarson Distribution and excretion of various mercury com pounds after single injections in poultry *Acta pharmac tox* 1968 a **26** 259—272

Swensson Å & U Ulfvarson Distribution and excretion of mercury compounds in rats over a long period after a single injection *Acta pharmac tox* 1968 b **26** 273—283

Takeda Y T Kunugi O Hoshino & T Ukita Distribution of inorganic aryl and

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Takeuchi T N Morikawa, H Matsumoto & Y Shiraishi A pathological study of Minamata Disease in Japan *Acta Neuropath* 1962, 2 40—57

Tejning S Biological effects of methyl mercury dicyandiamide treated grain in the domestic fowl Gallus gallus L VI Quantitative studies on the fate of ingested mercury Elimination in eggs and excrement deposition in the body and distribution in organs *Oikos* 1967 a Suppl 8 45—51

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Tejning S Biological effects of methyl mercury dicyandiamide-treated grain in the domestic fowl Gallus gallus L XI Juvenile development general health and mercury in organs in chicks hatched from eggs with presumed mercury contents of 0 2—0 4 mg *Oikos* 1967 d Suppl 8 75—76

Tejning S Biological effects of methyl mercury dicyandiamide-treated grain in the domestic fowl Gallus gallus L XII Effects on food consumption and egg laying symptomatology deposition of mercury in blood and organs, elimination of mercury in excrement and eggs and transportation to the plumage *Oikos* 1967 e Suppl 8 77—110

Tejning S Kvicksilverhalten i blodkroppar och i blodplasma hos arbetare exponerade for metylkvicksilverforening vid betning av utsade Rep no 67 02 10 from Yrkesmedicinska Kliniken Lasarettet Lund Sweden Lund 1968 a

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Tejning S & R Westerberg Alkyl mercury treated seed in food grain *Mercury in tissues and eggs from hens fed with grain containing methyl mercury dicyandiamide* *Poult Sci* 1964 43 6—11

The *mercury problem* Symposium concerning *mercury in* the environment Stockholm Januari 24—26 1966 *Oikos* 1967 Suppl 9

Tillander M Studies on *excretion rates of* organic mercury compounds in seal and fish Paper presented at Fifth *sympos um* on radioactivity in Scandinavia Helsinki *May 19—20 1969*

Torgeson D C Fungicides Vol I *Acad Press Inc* New York 1967

Toribara T Y & C P Shields. The *analysis* of submicrogram amounts of mercury in tissues *Am ind Hyg Ass J* 1968 29 87—93

U a T

Distribution of ^{203}Hg labelled determined by whole body auto-

—448 (In Japanese)

Ulfvarson U Betning av utsade. *Svensk Kem Tidskr* 1961 73 553—565

Ulfvarson U Distribution and *excretion of* some mercury compounds after long term exposure *Int Arch Gewerbepath Gewerbehyg* 1962, 19 412—422

Ulfvarson, U Determination of mercury in small quantities in biologic material by a modified photometric mercury vapor procedure *Acta chem scand* 1967 21 641—646

Ullberg, S Studies on the distribution and fate of S^{35} labelled benzylpenicillin in the body *Acta Radiol* 1954, suppl 118

Ullberg, S Autoradiographic studies on the distribution of labelled drugs in the body Second U N Int Conf Peaceful Uses of Atomic Energy 1958 24 248—254

Vesterberg, R & O Sjoholm On the determination of mercury and copper in biological material *Ark Kemi Miner Geol* 1946 22 A 1—9

Westermark, T, D Hagman A Vestermark & K Ljunggren Hittillsvarande utvecklingsarbete på den s k koncentrerande elektroforesmetoden for skiljande och påvisning av laddade kvicksilverforeningar i vattenlosning Nordiskt symposium kring kvicksilverproblematiken *Nord hyg Tidskr* 1969 50, 79—81

Westermark, T & B Sjostrand Activation analysis of mercury *Int J appl Rad at Isotopes* 1960, 9, 1—15

Westöö, Gunnel Determination of methylmercury compounds in foodstuffs I Methylmercury compounds in fish identification and determination *Acta chem scand* 1966, 20 2131—2137

Westöö, Gunnel Determination of methylmercury compounds in foodstuffs II Determination of methylmercury in fish egg meat and liver *Acta chem scand* 1967, 21 1790—1800

Williams E J Regression analysis John Wiley & Sons Inc London 1964 a 62—63

Williams E J Regression analysis John Wiley & Sons Inc London, 1964 b 60—61

Woeller F H Liquid scintillation counting of $C^{14}O_2$ with phenethylamine *Analyt Biochem* 1961 2 508—511

Wood J M F S Kennedy & C G Rosén Synthesis of methyl mercury compounds by extracts of a methanogenic bacterium *Nature* 1968 220 173—174

Yamashita M Distribution in organs and excretion of mercury on some experimental organic mercury compounds poisoning *J Japanese Soc intern Med* 1964 53 529—538 (In Japanese)

Yoshino Y T Mozai & K Nakao Distribution of mercury in the brain and its subcellular units in experimental organic mercury poisonings *J Neurochem* 1966 13 397—406

Zade A 1943 Swed Patent 109071 (Sept 16 1943)

Ulfvarson, U Determination of mercury in small quantities in biologic material by a modified photometric mercury vapor procedure *Acta chem scand* 1967, **21**, 641—646

Ullberg, S Studies on the distribution and fate of S[35]-labelled benzylpenicillin in the body *Acta Radiol* 1954, suppl 118

Ullberg, S Autoradiographic studies on the distribution of labelled drugs in the body Second U N Int Conf Peaceful Uses of Atomic Energy 1958, **24**, 248—254

Vesterberg, R & O Sjoholm On the determination of mercury and copper in biological material *Ark kemi Miner Geol* 1946, **22 A**, 1—9

Westermark, T, D Hagman, A Vestermark & K Ljunggren Hittillsvarande utvecklingsarbete på den s k koncentrerande elektroforesmetoden for skiljande och påvisning av laddade kvicksilverforeningar i vattenlosning Nordiskt symposium kring kvicksilverproblematiken *Nord hyg Tidskr* 1969, **50**, 79—81

Westermark, T & B Sjostrand Activation analysis of mercury *Int J appl Radiat Isotopes* 1960, **9**, 1—15

Westoo, Gunnel Determination of methylmercury compounds in foodstuffs I Methylmercury compounds in fish, identification and determination *Acta chem scand* 1966, **20**, 2131—2137

Westoo, Gunnel Determination of methylmercury compounds in foodstuffs II Determination of methylmercury in fish, egg meat, and liver *Acta chem scand* 1967, **21**, 1790—1800

Williams, E J Regression analysis John Wiley & Sons, Inc London, 1964 a 62—63

Williams, E J Regression analysis John Wiley & Sons Inc London, 1964 b 60—61

Wöeller, F H Liquid scintillation counting of $C^{14}O_2$ with phenethylamine *Analyt Biochem* 1961, **2**, 508—511

Wood, J M, F S Kennedy & C G Rosén Synthesis of methyl mercury compounds by extracts of a methanogenic bacterium *Nature* 1968, **220** 173—174

Yamashita, M Distribution in organs and excretion of mercury on some experimental organic mercury compounds poisoning *J Japanese Soc intern Med* 1964, **53**, 529—538 (In Japanese)

Yoshino, Y, T Mozai & K Nakao Distribution of mercury in the brain and its subcellular units in experimental organic mercury poisonings *J Neurochem* 1966, **13**, 397—406

Zade, A 1943 Swed Patent 109071 (Sept 16 1943)

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Interindividual Differences in Pharmacokinetics in Man

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SYMPOSIUM ON CLINICAL PHARMACOKINETICS

Interindividual Differences in Pharmacokinetics in Man

Moderator FOLKE SJÖQVIST

Balzar Alexanderson (Department of Pharmacology, Division of Clinical Pharmacol[illegible] Sweden): PHARMACO-
KINETIC S[illegible] INFLUENCE OF
GENETIC [illegible]

After repetitive oral administration of a constant dose of nortriptyline (NT) every 8 hours the plasma concentration (conc.) of the drug increases successively until a steady-state (ss) is achieved. The ss-conc. of NT in plasma varies at least ten-fold between patients treated with the same dose (in mg/kg).

Detailed pharmacokinetic investigations suggest that the ss-plasma conc. of NT or DMI (desmethylimipramine) is determined mainly by their rate of metabolism. Thus, only small interindividual differences were found in the plasma protein binding, degree of absorption and renal elimination of NT in man (Sjöqvist et al. 1969).

Studies in twins (Alexanderson et al. 1969) show that most of the variability in NT ss plasma conc. between persons who have not received other drugs is genetically determined. Exposure to other drugs also influences the ss-plasma conc of NT, which in a given patient may therefore be determined by a resultant of genetic and environmental factors. Twins who were on concomitant treatment with drugs containing barbiturates had considerably lower ss-plasma conc. of NT than the untreated control twin.

The ss-plasma conc of a drug is directly proportional to the maintenance dose and the half-time for elimination from plasma (PT 1/2) and is inversely proportional to the dosage interval and the apparent volume of distribution (V_d)

In order to elucidate the relative importance of the PT 1/2 and the apparent V_d in determining the ss plasma conc. of NT we have performed pharmacokinetic studies in seven healthy volunteers given single and repetitive oral doses of NT before and after treatment with phenobarbital. The following points will be discussed

a) The elimination curve of NT from human plasma after single oral doses.
b) The relationship between the PT 1/2 of NT determined after a single or oral dose and that calculated from the decline in plasma conc. after ss has been established and drug administration stopped (SS T 1/2).
c) The effect of phenobarbital treatment for three days on V_d and PT 1/2 of NT and the relationship between the "phenobarbital PT 1/2" and the SS T 1/2.

Accumulating evidence indicates that the pharmacologic effects of NT are directly related to its ss-conc. in plasma (Freyschuss et al. 1970). It therefore becomes important to recognize factors determining this pharmacokinetic parameter In this regard NT may be considered as a model for a great number of currently used drugs.

AND ME... MICROSOMES.

Oxidation and conjugation of drugs have usually been studied separately. In liver microsomal preparations only oxidated unconjugated *metabolites of* desmethylimipramine (DMI) occur unless UDPGA is added to the system. In the presence of UDPGA, however, unconjugated hydroxylated metabolites are further metabolized by conjugation with glucuronic acid, and the total rate of metabolism is increased. These findings indicate that under certain circumstances accumulation of hydroxylated unconjugated products can inhibit the m[illegible]
showed that [illegible]
than DMI, [illegible]
these two compounds to cytochrome P-450 was also studied. Both gave rise to so-called type I spectra and 2-OH-DMI inhibited the binding of DMI to the cytochrome competetively. This suggests that 2-OH-DMI inhibits the metabolism of DMI by occupying a site on cytochrome P-450.

Another interesting finding was that an apparent steady-state level of the intermediate hydroxylated unconjugated metabolites could be produced in the incubation mixture in the presence of UDPGA. A similar situation may occur in vivo.

The potential usefulness of the described incubation system for human pharmacologic studies will be discussed.

This study was supported by grants from the Swedish Medical Research Council (B70-13x-2471-03, B70-21x-1021-05) and the National Institutes of Health, Bethesda, U.S.A. (GM 13978-05).

REFERENCES

Alexanderson, B., F. Sjöqvist & D. A. Price Evans: Brit. med. J. 1969, 4, 764-768.
Freyschuss, U., F. Sjöqvist & D. Tuck: Pharmacol. clin. 1970, 2, 72-78.
Sjöqvist, F., W. Hammer, O. Borgå & D. L. Azarnoff: Proc. Coll. Int. Neuropsychopharmacol. Tarragona, Spain, April 1968. Excerpta med. Int. Congr. Ser. No. 180, 1969, 128-136.

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Björn Beermann, Kjell Hellström and Anders Rosén (Laboratory of Clinical Pharmacology and Department of Medicine, Serafimerlasarettet, Stockholm, Sweden): THE GASTROINTESTINAL ABSORPTION OF FOUR ANTICHOLINERGIC DRUGS IN MAN.

Healthy subjects were provided with a double lumen polyvinyl tube that was allowed to pass to desired levels of the small intestine before the start of the experiment. Water solutions of tritiated atropine, methylatropine, butylscopolamine (Buscopan) and Acabel together with polyethylene glycol (PEG, nonabsorbable marker) were administered orally or by intrajejunal infusion.

Intestinal aspirates obtained during the first hours were submitted to high voltage electrophoresis and paper chromatography. There was no evidence of a disposition of the labelled drugs.

The ratio between the amount of radioactivity per mg PEG of intestinal aspirates and that of the test solution demonstrated great differences in the absorption of the drugs. Atropine was almost completely absorbed, while the uptake of the three quarternary ammonium compounds in no instance exceeded 20 per cent of given dose. The degree of absorption of each of the four drugs showed only small interindividual differences.

The maximal plasma levels of radioactivity reflected the efficiency of the absorption of the drugs. Accordingly, the highest plasma level was seen after the administration of ^{3}H-atropine. No radioactivity was detected in the plasma of the subjects who received ^{3}H-butylscopolamine, the drug most poorly absorbed.

Gunnar Boman, Olof Borgå, Åke Hanngren, Anna-Stina Malmborg and Folke Sjöqvist (Departments of Thoracic Medicine and Bacteriology, [illegible] and Department of Pharmacology, Division of [illegible]

[illegible] AZID.

We have studied possible interactions between rifampicin (RMP) p amino-salicylic acid (PAS) and isoniazid (INH) with the following methods. All [illegible]

or in combination [illegible] h RMP also at 8 hours) PAS and INH in plasma were analyzed spectrophotometrically and RMP in serum microbiologically using Sarcinea lutea.

The simultaneous administration of PAS and INH resulted in significantly [illegible] INH. [illegible] med after i v. infusion of PAS that decelerated the disappearance of INH from plasma (after oral administration) (Hanngren et al. 1970)

PAS profoundly influenced the absorption of RMP when the two drugs were given together The peak concentrations of RMP appeared later and reached only about half the ordinary values Therefore, the serum concentration curves appeared extremely flattened out The reverse interaction also seemed to occur, i e , that RMP influenced the absorption of PAS However in this case the peak concentration of PAS was generally shifted to an earlier time point (from 6 hours to 2 hours) after administration, i e , the absorption process of PAS seemed to be speeded up by RMP

We have not been able to demonstrate any influence of RMP on the absorption or metabolism of INH Both plasma levels and half-lives were unchanged in this combination Nor was there any indication of an influence of INH on the absorption of RMP However the half-life of RMP was shorter in some subjects when INH was given at the same time If the material is divided into rapid and slow inactivators of INH only the 'slow' group is affected, the mean half-life of RMP decreasing from 4.6 to 3.6 hours

REFERENCE

Hanngren, Å O Borgå & F. Sjöqvist *Scand. J. respir. Dis.* 1970. In press

This study was supported by grants from Svenska Nationalföreningen mot Hjärt och Lungsjukdomar and the National Institutes of Health, Bethesda, U S A (GM 13978-05).

Olof Borgå and Per K. M. Lunde (Department of Pharmacology Division of Clinical Pharmacology, Karolinska Institutet, Stockholm, Sweden, and Institute of Pharmacology, University of Oslo, Norway) PROTEIN BINDING OF NORTRIPTYLINE AND DIPHENYLHYDANTOIN IN MAN.

We have investigated the plasma levels of the tricyclic antidepressant drug nortriptyline (NT) and the antiepileptic drug 5,5-diphenylhydantoin (DPH) in unbound form.

The plasma concentration of NT observed following usual therapeutic doses in man is 10 - 200 mµg/ml and its apparent volume of distribution is about 20 L/kg. Equivalent values for DPH are 1 - 50 g/ml and 0.50 L/kg. This suggests that the tissue binding of NT is much more extensive than that of DPH.

Using an ultrafiltration method at room temperature and therapeutic plasma concentration of drug, we found 5.5 ± 0.6 % of the NT (Borgå et al. 1969) and 7.4 ± 0.7 % (mean ± S.D.) of the DPH in human plasma in the unbound form (Lunde et al. 1970). The concentration of drug in the cerebrospinal fluid (CSF) approximates the concentration of unbound drug in the plasma during steady-state conditions. The concentration of NT in the CSF was 3 - 11 % of the simultaneously measured plasma concentration. This amount is near the sensitivity limit for the assay method. The concentration of DPH in the CSF was about 10 % of the plasma level (Berlin & Lund) which is similar to the fraction of unbound DPH observed at 37 °C (10.4 ± 0.4 %)

By an in vitro procedure utilizing isolated rat iris (rich in adrenergic neurons), the uptake of noradrenaline has been studied in the presence of NT (Borgå et al. 1970). The NT concentration which inhibited the uptake of noradrenaline by 50 % when plasma was the incubation medium was ten times the NT concentration needed when Krebs-Ringer solution was used This indicates that only 10 % of the NT in the plasma was in the unbound form. Experiments were performed at 37 °C

The unbound fraction of NT was nearly constant over a wide range of concentrations (therapeutic as well as toxic). The same was true for DPH.

We have also considered the possible importance of displacement of NT from plasma proteins by other drugs. Earlier in vitro work from this laboratory with desmethylimipramine, another tricyclic drug with similar structure, showed very small such effects (Borgå et al. 1969) Since plasma contains only a minute fraction of the total NT in the body (a few percent) displacement of bound NT from plasma proteins should not be expected to cause any significant increase of the drug level in other tissues It is also evident that the free NT level in the body is "buffered" by the extensive amount of drug bound to tissue. An increase in the absolute free concentration in plasma will be rapidly lowered by redistribution of NT to the tissues until the free plasma level again equals the free tissue level DPH was not significantly displaced from plasma protein by five other antiepileptic drugs in vitro. Phenylbutazone and salicylic acid at therapeutic concentrations caused a 2 - 3 fold increase in the free DPH concentration (Lunde et al 1970). Since DPH normally has a relatively small volume of distribution the observed effects may have therapeutic consequences

REFERENCES

Berl n A & L Lund Unpublished observations
Borgå O D L Azarnoff G Plym Forshell & F Sjöqvist Biochem Pharmacol 1969 18 2135 2143
Borgå O B Hamberger T Malmfors & F Sjöqvist Clin Pharmacol Ther 1970 In press
Lunde P K M A Rane S J Yaffe L Lund & F Sjöqvist Clin Pharmacol Ther 1970 Subm tted for publication

This study was supported by grants from the Swedish Medical Research Council (B70 21x 1021 05) and the National Institutes of Health Bethesda U S A (GM 13978 05)

Lars Lund (Departments of Neurology and Pharmacology, Division of Clinical Pharmacology, Karolinska Institutet, Stockholm, Sweden):
EFFECT AND SIDE EFFECTS OF DIPHENYLHYDANTOIN (DPH) IN RELATION TO PLASMA CONCENTRATIONS.

The plasma concentration of DPH (measured by the method of Dill) was determined in 148 ambulatory patients with grand mal- or mixed epilepsy. DPH was the only antiepileptic medication. In connection with a routine visit to the outpatient clinic the fit frequency during the last two months was noted as well as symptoms of DPH-intoxication. The plasma sample was then taken.

The material was divided into five groups according to the fit frequency. There was no significant difference in the prescribed dose of DPH between these groups. 85 % of patients with fits during the last two months had low concentration of DPH in plasma (less than 10 µ g/ml). The various explanations for this, e.g., unreliable drug intake, defect drug absorption and unusually fast drug metabolism will be discussed.

Eighteen patients had plasma levels above 20 µg/ml, which has been stated to be the upper limit of the therapeutic range. Subjective side effects (vertigo, fatigue) were noted in nine individuals with plasma levels from 20.4 to 63.2 µg/ml. Objective signs of DPH-intoxication (nystagmus, atactic gait, somnolence) were found in eight patients with DPH-concentrations from 29.1 to 63.2 µ g/ml. Pharmacokinetic observations elucidating the mechanism of DPH-accumulation will be presented. Of ten patients with concentrations of DPH above 30 µ g/ml three had no signs of intoxication. No signs of DPH-intoxication were found in the group with plasma concentrations below 20 µ g/ml.

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Anders Rane and Birgitta Jalling (Department of Pharmacology Division
[illegible] Institutet and Department of
[illegible]

In six infants 3 to 78 days old who were treated with diphenylhydantoin (DPH) in oral or intramuscular doses around 10 mg/kg/day because of convulsions the plasma concentrations of DPH were measured Unexpectedly they were only half of those previously observed in adults (treated with 5 mg/kg/day) and ranged between 1 0 8 3 μg/ml

One possible reason for this finding could be a lower degree of plasma protein binding of DPH This was studied, using an ultrafiltration technique and ^{14}C-labelled DPH

In cord plasma from 13 normal infants the binding capacity for DPH was lower (unbound DPH 10 6 $\pm$ 1 4 %) than in human adult plasma (unbound DPH 7 4 $\pm$ 0 7 %) at a plasma concentration of DPH of 16 μ g/ml In plasma samples from newborn infants the binding capacity was in the same range

There were greater interindividual variations in DPH-binding in cord plasma than in adult plasmas

The binding of DPH in hyperbilirubinemic infant plasma was less than in normal cord plasma There was a positive correlation between per cent unbound DPH and total bilirubin concentration The correlation coefficient was still greater between per cent unbound DPH and bilirubin/albumin ratio (Rane et al 1970)

REFERENCE

Rane A P K M Lunde B Jalling, S J Yaffe & F Sjöqvist. J Pediat 1970 Submitted for publication

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LECTURES

Marie Åsberg (Departments of Psychiatry and Pharmacology Division of Clinical Pharmacology Karolinska Institutet Stockholm Sweden) THE CORRELATION OF SUBJECTIVE SIDE-EFFECTS WITH PLASMA CONCENTRATIONS OF NORTRIPTYLINE

Plasma levels of tricyclic antidepressant drugs vary considerably between individuals receiving the same amount of drug The bearing of this variation on the occurrence of subjective side-effects was investigated in 40 psychiatric in-patients with depressive disorders Plasma levels were determined before and during 4 weeks of treatment with nortriptyline 50 mg t i d and patients were rated for subjective side-effects, the raters being unaware of the plasma level of the drug

There was a large variation in plasma levels between individual patients but in any given patient the plasma level tended to be constant over a period of time

The side-effects of nortriptyline diminished significantly with time and were in most cases absent during the fourth week of treatment

There was a significant positive correlation between plasma level of nortriptyline and subjective side-effects

The steady-state plasma level of a drug which is metabolized is usually a more important determinant for its effect than dosage since it reflects the amount of drug available for biological action

Very high plasma levels of nortriptyline should presumably be avoided since there is no evidence that they are needed for therapeutic effect and they are potentially harmful

The full account of this study will be published elsewhere (Åsberg Cronholm, Tuck & Sjöqvist)

This study was supported by grants from Carl-Bertel Nathhorst Foundation the Swedish Medical Research Council B70-21x 1021-05 the National Institutes of Health Bethesda U S A (GM 13978 05) the Association of the Swedish Pharmaceutical Industry and by funds from Karolinska Institutet

Reidun Ursin (Institute of Physiology University of Bergen Norway)

SLEEP AND SLEEP MECHANISMS

Sleep may be divided into 5 stages based upon the electroencephalogram (EEG) and recordings of the eye movements (Dement & Kleitman 1957) Stage 1 to 4 is defined from the EEG alone Stage 1 has a low amplitude EEG with no sleep spindles Stage 2 is mainly characterized by the sleep spindles (bursts of 12 14 c/sec activity on a low amplitude background) Stages 1 to 4 are defined from the EEG alone Stage 1 has a low amplitude waves (1 2 c/sec) These 4 stages together are often called slow wave sleep (SWS) or non REM (NREM) sleep The first stage is stage 1 REM The EEG is of the stage 1 type in addition there are rapid eye movements (REMS) This stage is therefore more often called REM sleep (other terms low voltage fast sleep activated sleep paradoxical sleep and D state)

Several physiological characteristics change during slow wave sleep and REM sleep (for references see Ursin 1967) During SWS muscle tonus and spinal reflexes are decreased but present Respiration is slow deep and regular Heart rate and blood pressure are lower than during the waking state The changes are usually more pronounced during stage 3 and 4 than during stage 2 During REM sleep muscle tonus is abolished in certain muscular groups but bursts of muscular twitches are seen in the face and limbs Spinal reflexes also tend to be abolished Respiration heart rate and blood pressure are characterized by its variability Penile erections accompany 95 % of all REM sleep periods in males from birth to old age

Dreams involving vivid sensory imagery are recalled on awakenings from REM sleep Recall of mental activity more like thinking may be obtained from SWS awakenings especially from stage 2

SWS and REM sleep alternate throughout the night in a cyclic fashion A REM sleep period starts approximately each 90 min in adults In the first half of the night the REM periods are shorter and are separated mainly by stages 3 and 4 Towards the end of the night they are longer and are separated mainly by stage 2 sleep

There seems to be a physiological need for deep SWS (stage 3 and especially stage 4) and for REM sleep Selective deprivation results in a compensatory rebound on the first undisturbed night After total sleep deprivation there is a deep SWS rebound the first night and if the deprivation was long enough a REM sleep rebound in the second night

Total sleep length is age dependent (Webb 1969) The sleep time of young adults may vary from less than 6 hours to more than 9 hours The wide individual differences may be due to constitutional factors learning physiological variables and environmental factors The per cents of a night spent in the different stages are as follows (young adult men Williams et al 1964) Stage 1 5 % stage 2 49 % stage 3 8 % stage 4 13 % stage 1 REM 24 % This sleep pattern also shows great differences among subjects but is highly consistent within an individual

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The concept of sleep and wakefulness as a function of the activity of the brain stem reticular formation needs some supplement. Actively synchronizing areas have been described in the forebrain and in the lower brain stem. Recent investigations suggest that the monoamines serotonin and noradrenaline are involved in sleep regulation. According to Jouvet (1968), lesion of the nucleus raphe pontis in cats - with 5-hydroxytryptamine-containing neurons - drastically reduced both SWS and REM sleep. Locus coeruleus of pons, which contains monoamino-oxidase (MAO) and noradrenaline, is believed to participate in the triggering of REM sleep. Pharmacological agents that affect monoamine metabolism may also affect sleep. P-chlorophenylalanine blocks SWS and REM sleep in cats In humans, REM sleep is increased by reserpine, and reduced by MAO-inhibitors and imipramine with no rebound on withdrawal.

Several of the commonly used sedatives and hypnotics alter the sleep pattern (Kales 1969). The investigations have been concentrated mainly on the effects on REM sleep. A common effect is a reduction of this type of sleep with a *compensatory rebound on withdrawal. This is the case for most barbiturates* glutethimide, methyprylon, meprobamate and alcohol. The barbiturates, glutethimide and alcohol also tend to reduce SWS stage 4. The benzodiazepines does not seem to affect REM sleep per cent but may reduce SWS stage 4 especially with chronic use. The sleep pattern observed following administration of chloral hydrate does not differ much from the normal.

REFERENCES

Dement, W. & N. Kleitman: Electroenceph. clin. Neurophysiol. 1957, 9, 673-690.

Jouvet, M.: Advances in Pharmacology, Vol. 6B. Academic Press, New York, 1968, 265-279.

Kales, A.: Ann. intern. Med. 1969, 70, 591-614.

Ursin, R.: T. norske Lægeforen. 1967, 87, 1736-1741.

Webb, W. B.: Sleep: Physiology and Pathology. Lippincott, Philadelphia, 1969, 53-65.

Williams, R L , H W Agnew Jr. & W B Webb: Electroenceph clin. Neurophysiol. 1964, 17, 376-381.

I ar Oye (Inst tute of Pharmacology Un ers ty of Oslo Norway)

THE PRESENT STATE OF THE SECOND MESSENGER THEORY

Nucle c ac ds and nucleot des are nvol ed n informat on transm ss on in b olog cal systems n many ways Messages are coded n DNA and trans ferred by RNA ATP ADP AMP and other nucleot de coenzymes serve as feed back s gnals for metabol c regulat on Accord ng to the second mes senger theory cycl c 3 5 AMP serves as an ntracellular mediator n the act on of several hormones and transm tters

In some organ sms l ke the sl me mold (d ctyostel um) cycl c 3 5 AMP med ates informat on from one cell to another In some organ sms like e col informat on about unspec f c changes n the env ronment s conveyed by cycl c 3 5 AMP to ntracellular systems nvol ed n prote n synthes s and growth In ... of cy l c 3 5 AMP s apparently conf ned to ntracellul ... m tters) transm ss on ...
of hormone or transm tter (f rst messenger) Th s messenger reacts w th appropr ate receptors n the target cells to produce a new molecular spec es (the second messenger) wh ch acts on several ndependent cellular processes e ther d rectly or through subsequent messengers or react on sequences

Other cycl c nucleot des ha e been found but cycl c 3 5 AMP s so far the only one def n tely known to serve as a second messenger n hormone action The fact that the same molecular species serves as a messenger for several d fferent hormones nd cates that the spec f c ty of these hormones resides n the conformat onal relat onsh p between the hormone and receptor s tes n the target cells The nature of the phys ological response depends on the func t onal apab l es of the target cells The second messenger s a relat ely unspec f c l nk between recep or and response

T ansm ss on of nforma on by a second messenger offers the poss b l ty of ampl f at on o the ext acellular s gnal (one hormone molecule causing the produ t on of se eral messenger molecules) and of super mposed control of nfo ma on nput w th n the target cells Drugs ma act by st mulat ng or n h b ng s nthes s or breakdown of the second messenger or by m m ck ng o blo k ng ts act ons

A ap dl g ow ng body of e dence supports the val d ty of the second mes senge t eo y n the ac on of a number of hormones concerned w th the re gula o of metabol sm and secret on The role of the second messenger in muscle and ne e s not so well understood at present Exper ments with he so a ed a hea t ser ng to llustrate th s po nt w ll be d scussed The ha a sm te s st mulate adenyl cyclase n ner e and muscle does ne ess r mea that c cl c 3 5 AMP s an obl gatory second messer he x a on (or n b t on) produced by these transm tters It s poss b he of t e seco d messenger n ner e and muscle is to n ed t r ab ut the fu ct onal state of the cells to more bas c b o h n a s s n er d a th e ergy s pp and prote n synthesis

COMMUNICATIONS

LET

There is evidence that certain analgesic drugs inhibit the monoamine uptake into neurons (Carlsson & Lindqvist 1969) and into blood platelets (Ahtee & Saarnivaara 1970) In the present communication further studies on this uptake inhibition are reported

After 15 min preincubation of human blood platelet suspension or rabbit platelet rich plasma with various analgesics 5HT (10^{-5} M) or ^{3}H 5HT (10^{-6} M) or metaraminol (MA) (3×10^{-6} M) were added and the incubation at 37 °C under carbogen was continued for 1 hour

From the eleven drugs studied methadone, pentazocine, anileridine and pethidine most potently inhibited the 5HT uptake into human blood platelets. Already 10^{-5} M of these drugs significantly inhibited the 5HT uptake and this inhibitory effect was dose-dependent At the concentration of 10^{-4} M methadone pentazocine and anileridine inhibited 80 to 100 % of 5HT uptake whereas at this concentration pethidine inhibited 38 % of 5HT uptake and dextropropoxyphene 26 % respectively These drugs also released 5HT from platelets The most potent releasing drug was methadone which released 27 % of platelet 5HT at 10^{-4} M concentration in 1 hour whereas 10^{-3} M of pethidine was needed for a similar release Morphine, codeine, apomorphine and nalorphine were almost inactive whereas thebaine at 10^{-4} M concentration inhibited the uptake by 46 % but it also released 15 % of platelet 5HT at this concentration

We also studied the effect of these drugs on the uptake of MA into rabbit blood platelets The uptake of MA into platelets was directly proportional to the extracellular concentration of MA up to the concentration of 3×10^{-5} M At this concentration MA released about 10 % of platelet 5HT. Lower concentrations of MA did not release any 5HT from platelets whereas higher concentrations of MA released it dose-dependently The most potent 5HT uptake inhibitors were also the most potent MA uptake inhibitors and they inhibited the MA uptake into platelets significantly more than equimolar concentrations of imipramine and desipramine

These results could explain the toxic reactions seen when analgesics are combined with drugs which increase the synaptic monoamines

REFERENCES

Ahtee L & L Saarnivaara Scand J clin Lab Invest 1970, 25 (Suppl 113) 19

Carlsson A & M Lindqvist J Pharm Pharmacol 1969 21 460-464

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Laake (Norwegian Defence
icology Kjeller Norway)

A BARRIER TO QUARTERNARY NITROGEN COMPOUNDS

Pharmacology of the ret na has been a rather neglected field It is therefore natural that the access of drugs to the retina has attracted limited interest

Ontogenetically retina is a part of the brain The blood brain barrier (BBB) might therefore be supposed to encompass the retina

Intravenously administered horse radish peroxidase (mol w ≈ 40 000) does not penetrate the BBB (Reese & Karnovsky 1967) whereas recent electron microscopic studies (Olsen 1970) indicate that the blood retina barrier (BRB) is permeable to this enzyme This indicates a difference between BBB and BRB with regard to pore size

The BBB is known to be slightly permeable to quarternary nitrogen com pounds To gain knowledge about the BRB in this respect the following ex periments were performed

An irreversible AChE inhibitor containing quarternary nitrogen (isopropyl ethylphosphonyl 2 acetyl 1 methylpyridinium oxime iodide 2 MPAM ES) was administered subcutaneously 0 325 mg/kg to six atropinized male rats Sixteen hours later the animals were sacrificed and ChE activities determined (according to Ellman et al 1961) in whole blood and homogenates from liver retina and the cerebral cortex the values being compared with those of six untreated control animals Blood and liver ChE activities were reduced to 26 ± 6 and 33 ± 11 % of the controls respectively the correspond ing values for brain and retina being 85 ± 26 and 87 ± 24 % (values are pre sented at P = 0 05 throughout)

To 12 atrop nized animals paraoxon 0 3 mg/kg was injected intravenously Starting 2 1/2 hours later three doses of pralidoxime methanesulphonate (P2S) 60 mg/kg each were given subcutaneously at one hour intervals to six animals the remaining six serving as controls The animals were killed 18 hours later The controls showed 37 ± 4 5 30 ± 3 9 and 36 ± 15 5 % of nor mal ChE activity in blood brain and retina respectively In the same se quence the values from the P2S treated animals were 93 ± 18 32 ± 4 5 and 83 ± 10 9 % showing a significant ChE reactivation in retina but not in brain

The results ind cate that BRB resembles BBB in being rather imperable to 2 MPAM ES In contrast BRB is easily permeable to P2S (which BBB is not) D fferent molecular sizes seem unlikely to explain this discrepancy perox dase penetration through BRB taken into account If the presented results should lead to any preliminary conclusion it might be that BRB has features n common with but is more permeable than BBB

REFERENCES

Ellman G L K D Courtney V Andres Jr & R M Featherstone B ochem Pharmacol 1961 7 88-95
Olsen B R Personal communicat on 1970
Reese T S & M J Karnovsky J Cell Biol 1967 34 204 217

N.-E. Andén, S. G. Butcher and J. Engel (Department of Pharmacology, University of Göteborg, Sweden): CENTRAL RECEPTOR ACTIVITY OF AMINES FORMED FROM L-DOPA AND SOME DOPA ANALOGS

Injection of L-3,4-dihydroxyphenylalanine (DOPA, 75 mg/kg i p 1 hour after nialamide 50 mg/kg i.p.) caused formation of dopamine (DA) and noradrenaline (NA) in the brain and spinal cord of rats also after pretreatment with reserpine plus α-methyltyrosine The DOPA-induced stimulation of central NA receptors was revealed as an increase in the hindlimb flexor reflex activity of spinal rats. The formation of NA, but not of DA, was blocked by a DA-β-hydroxylase inhibitor (FLA-63). Simultaneously, the increase in flexor reflex activity was severely reduced indicating only a slight action of DA on the central NA receptors. Central DA receptor activity was investigated by observing asymmetries in unilaterally striatotomized rats. After DOPA treatment, the rats turned to the operated side with a time course roughly correlated to the DA level.

The DOPA analogs DL-metatyrosine (150 mg/kg i.p. 1 hour after nialamide 50 mg/kg i.p.), DL-α-methyl-metatyrosine (800 mg/kg i.p.) and L-α-methyl-DOPA (400 mg/kg i.p.) were also decarboxylated and then partly β-hydroxylated to the corresponding amines in rats pretreated with reserpine. The amine formation from metatyrosine was at least as great as that from DOPA whereas the decarboxylation of the α-methylated amino acids was much smaller though more longlasting. Due to this slow decarboxylation, the β-hydroxylated amine derivatives of the α-methylated amino acids (metaraminol, α-methyl-NA) reached about the same levels as their non-α-methylated congeners (metaoctopamine, NA) Injection of metatyrosine to unilaterally striatotomized rats caused an asymmetry of almost the same magnitude as after administration of DOPA This effect and the accumulation of metatyramine had about the same duration. Treatment with α-methyl-DOPA or α-methyl-metatyrosine produced little or no turning of unilaterally striatotomized rats. The flexor reflex activity of spinal rats was markedly stimulated after injection of α-methyl-DOPA but was not significantly influenced after treatment with metatyrosine or α-methyl-metatyrosine. This increase was accompanied by an accumulation of α-methyl-NA. Pretreatment with FLA-63 markedly reduced both the flexor reflex activity and the concentration of α-methyl-NA.

In conclusion, DOPA causes central stimulation of both DA and NA receptors whereas α-methyl-DOPA and metatyrosine produce stimulation of mainly NA and DA receptors, respectively, and α-methyl-metatyrosine appears inefficient.

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Intraveno ... peroxidase (mol w ≈ 40 000) does not penet ... microsco ... is permeable to this enzyme This indicates a ... BRB with regard to pore size

The BBB is known to be slightly permeable to quarternary nitrogen compounds To gain knowledge about the BRB in this respect the following experiments were performed

An irreversible AChE inhibitor containing quarternary nitrogen (isopropyl ethylphosphonyl 2 acetyl 1 methylpyridinium oxime iodide 2 MPAM ES) was administered subcutaneously 0 325 mg/kg to six atropinized male rats Sixteen hours later the animals were sacrificed and ChE activities determined (according to Ellman et al 1961) in whole blood and homogenates from liver retina and the cerebral cortex the values being compared with those of six untreated control animals Blood and liver ChE activities were reduced to 26 ± 6 and 33 ± 11 % of the controls respectively the corresponding values for brain and retina being 85 ± 26 and 87 ± 24 % (values are presented at P = 0 05 throughout)

six animals the remaining six serving as controls The animals were ... ed 18 hours later The controls showed 37 ± 4 5 30 ± 3 9 and 36 ± 15 5 % of normal ChE activity in blood brain and retina respectively In the same sequence the values from the P2S treated animals were 93 ± 18 32 ± 4 5 and 83 ± 10 9 % showing a significant ChE reactivation in retina but not in brain

The results indicate that BRB resembles BBB in being rather imperable to 2 MPAM ES In contrast BRB is easily permeable to P2S (which BBB is not) Different molecular sizes seem unlikely to explain this discrepancy peroxidase penetration through BRB taken into account If the presented results should lead to any preliminary conclusion it might be that BRB has features in common with but is more permeable than BBB

REFERENCES

Ellman G L K D Courtney V Andres Jr & R M Featherstone Biochem Pharmacol 1961 7 88-95
Olsen B R Personal communication 1970
Reese T S & M J Karnovsky J Cell Biol 1967 34 204 217

N.-E. Andén, S. G. Butcher and J. Engel (Department of Pharmacology, University of Göteborg, Sweden): CENTRAL RECEPTOR ACTIVITY OF AMINES FORMED FROM L-DOPA AND SOME DOPA ANALOGS.

Injection of L-3,4-dihydroxyphenylalanine (DOPA, 75 mg/kg i.p. 1 hour after nialamide 50 mg/kg i.p.) caused formation of dopamine (DA) and noradrenaline (NA) in the brain and spinal cord of rats also after pretreatment with reserpine plus α-methyltyrosine. The DOPA-induced stimulation of central NA receptors was revealed as an increase in the hindlimb flexor reflex activity of spinal rats. The formation of NA, but not of DA, was blocked by a DA-β-hydroxylase inhibitor (FLA-63). Simultaneously, the increase in flexor reflex activity was severely reduced indicating only a slight action of DA on the central NA receptors. Central DA receptor activity was investigated by observing asymmetries in unilaterally striatotomized rats. After DOPA treatment, the rats turned to the operated side with a time course roughly correlated to the DA level.

The DOPA analogs DL-metatyrosine (150 mg/kg i.p. 1 hour after nialamide 50 mg/kg i.p.), DL-α-methyl-metatyrosine (800 mg/kg i.p.) and L-α-methyl-DOPA (400 mg/kg i.p.) were also decarboxylated and then partly β-hydroxylated to the corresponding amines in rats pretreated with reserpine. The amine formation from metatyrosine was at least as great as that from DOPA whereas the decarboxylation of the α-methylated amino acids was much smaller though more longlasting. Due to this slow decarboxylation, the β-hydroxylated amine derivatives of the α-methylated amino acids (metaraminol, α-methyl-NA) reached about the same levels as their non-α-methylated congeners (metaoctopamine, NA). Injection of metatyrosine to unilaterally striatotomized rats caused an asymmetry of almost the same magnitude as after administration of DOPA. This effect and the accumulation of metatyramine had about the same duration. Treatment with α-methyl-DOPA or α-methyl-metatyrosine produced little or no turning of unilaterally striatotomized rats. The flexor reflex activity of spinal rats was markedly stimulated after injection of α-methyl-DOPA but was not significantly influenced after treatment with metatyrosine or α-methyl-metatyrosine. This increase was accompanied by an accumulation of α-methyl-NA. Pretreatment with FLA-63 markedly reduced both the flexor reflex activity and the concentration of α-methyl-NA.

In conclusion, DOPA causes central stimulation of both DA and NA receptors whereas α-methyl-DOPA and metatyrosine produce stimulation of mainly NA and DA receptors, respectively, and α-methyl-metatyrosine appears inefficient.

Lars-Erik Appelgren (Department of Pharmacology, Royal Veterinary College, Stockholm Sweden) DISTRIBUTION OF LABELLED 5α-DIHYDROTESTOSTERONE IN MICE

showed a high and specific localization of radioactivity in ductus deferens (Appelgren 1969) and some of the radioactivity in ductus deferens behaved chromatographically as 5α-dihydrotestosterone (Appelgren 1970) To study the affinity of exogenous 5α-dihydrotestosterone to ductus deferens and other androgen dependent structures a whole body autoradiographic study according to Ullberg (1954) was performed after an i v injection of ^{3}H-5α-dihydrotestosterone As early as 2 minutes after injection the concentration of radioactivity was very high in vas deferens and in the interstitial cells of the testicles The liver, brain and adrenal cortex also showed high amount of ^{3}H In the prostates, however, rather low concentration of radioactivity was registered although these glands are known to convert testosterone to 5α-dihydrotestosterone to a large extent Twenty minutes after injection the highest amount of radioactivity was found in the liver intestinal contents salivary gland and ductus deferens in decreasing order Preliminary chromatographic experiments on chloroform extracts from testicles and ductus deferens after injection of 5α-dihydrotestosterone showed 3 major radioactive spots including the injected substance

The ability of vas deferens to specifically accumulate and metabolize exogenous 5α-dihydrotestosterone and testosterone supports earlier investigations by Hamilton et al (1969) showing the androgen dependent ability of vas deferens to synthetize steroids

REFERENCES

Appelgren L E Acta endocrin Kbh 1969 62 505-512
Appelgren L -E Biol Reprod 1970 In press
Gloyna R E & J D Wilson J clin Endocrin 1969, 29 970-977.
Hamilton D W A L Johns & D W Fawcett, Biol Reprod 1969, 1 167-184
Ullberg S Acta Radiol Stockh 1954, Suppl 118, 1-110.

This investigation was supported by the Swedish Medical Research Council and Knut och Alice Wallenbergs Stiftelse

R G G Andersson, L Lundholm and E Mohme-Lundholm (Department of Pharmacology, The Medical School, Linköping, Sweden)

ROLE OF Ca^{++} IN METABOLIC ACTIONS OF DRUGS IN INTESTINAL SMOOTH MUSCLE

The tension of the rabbit colonic muscle was increased by carbacholine ($1.5\cdot10^{-7}$ g/ml) and by K^{+}-ions (145 mEq/l), when in a Krebs-Henseleit solution the Na^{+}-ions were replaced by K^{+}-ions This effect was associated with a significant increase of the cyclic AMP content and phosphorylase a activity of the muscle There also was an increase of the hexose-phosphate contents but a significant decrease of the ATP and CrP contents In Ca^{++}-poor preparations both mechanical and metabolic actions induced by these drugs were completely eliminated Phenylephrine ($1\cdot10^{-6}$ g/ml), which relaxed the muscle via a specific stimulation of adrenergic alfa-receptors, reduced the cyclic AMP content, the phosphorylase a activity and the content of hexose-phosphates but increased the ATP content of the muscle These actions were blocked by dibenamine, an alpha-adrenergic blocking agent which by itself relaxed the muscle somewhat and reduced the phosphorylase a activity In the Ca^{++}-poor muscle contracted by Ba^{++}, phenylephrine had no relaxing action at all The action of phenylephrine on phosphorylase a activity was changed to a slight stimulating effect and the hexose-phosphate contents were slightly increased The ATP and CrP increasing actions were blocked in the Ca^{++}-poor preparation Ca^{++}-lack induced a decreased phosphorylase a activity in comparison with a normal muscle On addition of Ca^{++} or Ba^{++} to the Ca^{++}-poor muscle, the phosphorylase a activity increased significantly Isoprenaline in low concentrations selectively stimulated adrenergic β-receptors and relaxed the rabbit colonic muscle This effect was associated with an increased cyclic AMP content, a stimulation of the phosphorylase a activity and a decrease of the content of high energy phosphate compounds These effects could be reproduced by addition of cyclic AMP In Ca^{++}-poor muscle isoprenaline and cyclic AMP still significantly increased the phosphorylase a activity, but their reducing action on high energy phosphate compounds were blocked It is suggested that an increased cyclic AMP content and a stimulation of carbohydrate-metabolism in colonic muscle can be caused both by an adrenergic β-receptor stimulation and by contracting agents such as carbacholine and K^{+}-ions The first effect is Ca^{++} independent whereas the second is Ca^{++} dependent

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5α dihydrotestosterone is regarded as an androgenically active metabolite of testosterone in
Gloyna & Wilson 1969)
showed a high and spec
(Appelgren 1969) and su
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Appelgren L E Biol Reprod 1970 In press
Gloyna R E & J D Wilson J clin Endocrin 1969 29 970 977
Hamilton D W A L Johns & D W Fawcett Biol Reprod 1969 1 167 184
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This investigation was supported by the Swedish Medical Research Council and Knut och Alice Wallenbergs Stiftelse

R G ... and E Mohme-Lundholm (Department ... School Linköping Sweden)

ROLE ... [illegible] TIONS OF DRUGS IN INTESTINAL SMOOTH MUSCLE

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Sten-Magnus Aquilonius (Research Institute of National Defence, Dept 1, Sundbyberg Sweden) Jan Schuberth (Psychiatric Research Center, Ulleråker Hospital Uppsala Sweden) and Anders Sundwall (Department of Pharmacology and Toxicology Kabi Stockholm Sweden) STUDIES ON CHOLINE IN CEREBROSPINAL FLUID

Choline (Ch) is proximate precursor in acetylcholine (ACh) synthesis and transmittor metabolite Intravenously injected labelled Ch is transported into brain and rapidly incorporated in ACh of nerve endings (Schuberth et al 1970) Since Ch concentration in cerebrospinal fluid (CSF) is very low a significant part of CSF Ch may be derived from ACh breakdown in brain even if some of the Ch split off may be recaptured In that case CSF-Ch concentration would reflect ACh turnover This hypothesis is supported by reports of decreased cisternal CSF-Ch in dogs following pentobarbital (Bowers 1967) which reduces ACh turnover and increased spinal CSF-Ch in humans during amphetamine intoxication (Jönsson et al 1969)

Serial sampling of ventricular and cisternal CSF was performed in conscious unrestrained dogs through permanently implanted cannulas 0 1 ml samples were withdrawn and Ch determined by the Ch acetyltransferase method Ch concentration in lateral ventricle was 4 6 ± 0 25 nmoles/ml and in cisterna 1 9 ± 0 26 nmoles/ml The ventriculo-cisternal concentration ratio of 2 4 is of the same magnitude as that reported in different species for the monoamine metabolite 5-HIAA (Andersson 1968) and indicates that most of the Ch is delivered to CSF withinthe ventricles and that Ch is removed from CSF during passage to cisterna magna

Following oxotremorine known to decrease ACh turnover in brain a 50 % decrease in Ch concentration in lateral ventricle was observed while decrease in cisternal values was nonsignificant Results with other drugs known to alter cholinergic activity (atropine pentobarbital amphetamine) were contradictionary and no significant effect upon Ch concentration was seen High affinity of Ch to a transport system in choroid plexus (Tochino & Schanker 1964) may account for the difficulty to alter CSF-Ch concentration Efforts to block plexus uptake of Ch are in progress

REFERENCES

Andersson H Dev Med Child Neurol 1968, Suppl 15, 58-61
Bowers M B Life sci 1967 6 1927-1933
Jönsson L E J Schuberth & A Sundwall Life sci 1969, 8, 977-981
Schuberth J B Sparf & A Sundwall J Neurochem 1970 In press
Tochino Y & L S Schanker Pharmacologist 1964, 6, 177

Sten-Magnus Aquilonius Lars Frankenberg, Birgitta Lundholm Karl-Erland Stensiö and Birger Winbladh (Research Institute of National Defence, Dept 1, Sundbyberg, Sweden) SOME EFFECTS OF A CHOLINE ACETYLTRANSFERASE INHIBITOR

Specific inhibitors of choline acetyltransferase (ChAT) would be valuable tools in the study of cholinergic mechanisms In vitro potent reversible and irreversible ChAT inhibitors have been developed but pharmacological data are yet lacking The trans isomer of 4(β(1-naphtyl)vinyl) pyridine hydrochloride shown to be a potent reversible inhibitor in vitro with I 50 $3x10^{-5}$M (Smith et al 1967) was synthetized The essentially inactive cis isomer was derived upon UV illumination of the trans isomer in solution and isolated by chromatographic separation

Experiments were performed on the ischiadicus gastrocnemius preparation in the cat Intravenous injection of the trans isomer (5-15 mg/kg) was without effect while close arterial injection produced a slowly progressing decrease in twitch amplitude with a concomitant slow rise in basal tension and a marked drop in muscle temperature Tetanic stimulation gave a poorly sustained contraction and posttetanic stimulation vanished However, as the cis isomer produced identical effects these cannot be ascribed to ChAT inhibition The results rather resemble the effect on muscular metabolism described for monoiodoacetic acid by Lundsgaard (1930) Experiments with the quaternary ethanol derivative recently described as a potent ChAT inhibitor in vitro (Cavallito 1970) are in progress This compound seems to lack the above described effects but produces reversible inhibition of neuromuscular function

REFERENCES

Cavallito, C J Int symposium on the effects of drugs on cholinergic mechanisms in the CNS 1970 Unpublished
Lundsgaard, E Biochem Z 1930 217 162-177
Smith, J C , C J Cavallito & F F Foldes Biochem Pharmacol 1967, 16, 2438-2441.

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CHOLINE IN CEREBRO...

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Bowers M B Life sci 1967 6 1927-1933
Jönsson L E J Schuberth & A Sundwall Life sci 1969, 8 977-981
Schuberth J B Sparf & A Sundwall J Neurochem 1970 In press
Tochino Y & L S Schanker Pharmacologist 1964, 6, 177

P Arvela and N T Kärki (Department of Pharmacology, University of Oulu, Finland): THE EFFECT OF CERIUM ON DRUG METABOLIZING ACTIVITY IN RAT LIVER

Some of the rare earths when injected intravenously into rats and other species produce fatty liver degeneration Earlier investigations also show that there are changes in the endoplasmic reticulum which manifest primarily as dilatations of the cisternae and dissociation of ribosomes We have been interested to know if the morphological changes are accompanied with the impairment of the drug metabolizing capacity of rat liver

Following a single intravenous injection of 2 mg cerium/kg as a solution of the chloride at pH 3 5 - 4 0 rats were decapitated after 1, 2, 3, 6 and 16 days The 20 % liver homogenates were made in 1 15 % ice-cold KCl The 15 000 x g supernatant of homogenates was used as enzyme preparation and its activity to oxidate hexobarbital and to dealkylate N-methylaniline was measured

It could be stated that cerium reduced the capacity of the liver to metabolize both used test-substances The decrease was most pronounced after three days of the cerium injection The hexobarbital metabolizing activity was decreased to 31 2 % and the N-methylaniline dealkylating activity to 57 0 % of control respectively The metabolizing capacities were restored completely in 16 days In accordance to other studies it could be stated an increase in the plasma free fatty acid level and a decrease in blood glucose These changes were most prominent on the second day after cerium injection and were restored in six days

Our studies showed that cerium impairs the drug metabolism in rat liver and this parallels to the earlier findings that rare earths causes subcellular changes in the liver cells

Ernst Bárány (Institute of Pharmacology, University of Uppsala, Sweden)

RELEASE OF A NON-ADRENERGIC SMOOTH-MUSCLE ACTIVE COMPOUND FROM DEGENERATING SYMPATHETIC NERVE TERMINALS

About 14 hours after r
release of noradrenal
starts and is seen as
tation around 20 hours after denervation Surprisingly, this peak is accompanied by a hyperaemia of the iris, not by vasoconstriction as one would have expected Bretylium injected before the degeneration release is known to delay it In the present system, 10 mg/kg at the time of denervation and 10 hours later cause a 5 hour delay of the mydriasis but a 9 hour delay of the hyperaemia This indicates that the hyperaemia is not secondary to the release of noradrenaline

The hyperaemia is not preventable by reserpine or phenoxybenzamine and not affected by phentolamine propranolol, butoxamine, spiroperidol, chlorpromazine, lidocaine or atropine

While during the early stages the mydriasis is fully reversible by phentolamine, the late stages in the presence of hyperaemia, are only partly reversible

It seems therefore that a substance is released from the degenerating nerve endings that acts on the vessels and the smooth muscle of the iris This substance is released after the release of noradrenaline

(*Work together with* G Treister Supported by NIH grant EY-00231 and grant K69-14X-723-040 from the Swedish Medical Research Council)

Anders Bergendorff and Börje Uvnäs (Department of Pharmacology Karolinska Institutet Stockholm Sweden) STORAGE OF 5-HYDROXY-TRYPTAMINE (5-HT) IN MAST CELLS

Histamine and 5-HT-containing granules have been isolated from rat mast cells lysed in deionized water The granules consist essentially of a heparin-protein complex When suspended in sodium chloride solution the granules release their amines and take up sodium These granules may be depleted of their sodium by repeated suspension in deionized water

The in vitro uptake of 5-HT and histamine by such isolated depleted mast cell granules was similar The uptake is related to the extragranular amine concentration and the pH of the incubation media in a way that suggests an electrostatic linkage to carboxyl groups of the heparin-protein complex The 5-HT taken up by the granules can be released on suspension in sodium chloride solution in accordance with the theory that 5-HT - like histamine - is displaced by sodium at ionic sites in the granules

' (Department of Pharmacology
of Oslo Norway) A COMPARISON OF
LYCOSIDES OF THE ISOLATED
INTESTINE FROM THE RAT AND THE GUINEA PIG

A method described by Bieltvedt & Briseid (1966) and Bieltvedt (1967) for determination of the inhibition by cardiac glycosides of the isolated histamine-stimulated guinea pig ileum was modified to estimate the inhibition of the acetylcholine stimulated *rat jejunum* The glycoside concentrations caus ng 50 % inhibition of submaximal isotonic contractions were determined and the procedure was based on 20 minute contact periods between the muscle preparations and the cardio-active substances and then 22 minute equilibration periods with chemical stimulation The degree of inhibition was found to depend on the potassium content of the Tyrode solutions used In a low potassium Tyrode solution (1 8 mM KCl) the inhibition of the rat intestine by cardiac glycosides was found to be of the same order of size as the inhibition of the guinea pig intestine When the potassium chloride concentration was increased to 2.7 mM, the well known species difference was observed the concentration of cardiac glycoside required for inhibition of the rat intestine being about 25 times higher than that required for inhibition of the guinea pig intestine The

of the isolated intestine preparations a theory of qualitative differences between the transport enzymes in the two species is advanced In the rat intestine the presence is suggested of one ATPase being rather resistant to inhibition by cardiac glycosides but sensitive to a reduction in potassium concentration in addition to a glycoside susceptible ATPase present also in the guinea pig intestine

REFERENCES

Bieltvedt G Acta pharmacol et toxicol 1967 25 80 86
Bieltvedt G & K Briseid Acta pharmacol et toxicol 1966 24 157-168

Ole Jacob Broch Jr. and Hans Cato Guldberg (Department of Pharmacology, University of Bergen, Norway): A FLUOROMETRIC METHOD FOR THE DETERMINATION OF COMT ACTIVITY IN BRAIN.

A sensitive and specific spectrophotofluorometric method for the determination of catechol-0-methyl transferase (COMT) activity in crude homogenates from brain was developed. The method is based on the enzymic conversion, in the presence of S-adenosyl methionine (SAM) and magnesium ions, of 3,4-dihydroxyphenylacetic acid to 4-hydroxy,3-methoxyphenylacetic acid (Assicot & Bohuon 1969). The end-product was separated by paper chromatography and, after the development of a fluorophor, determined fluorometrically.

After homogenization of the tissue in phosphate buffer the enzyme activity could be determined accurately in amounts of brain tissue down to 30 mg in wet weight. The enzyme activity was found to be related linearly to the amount of tissue as well as to the time of incubation. Km was determined in whole homogenates from rat brain using a Lineweaver-Burk plot and was found to be 2.0×10^{-4} M. This value is in good agreement with that of earlier publications using a purified enzyme preparation.

Homogenization in distilled water followed by centrifugation (30 000 g) gave a precipitate with negligible activity, while the activity in the supernatant was of the same order as that from a whole homogenate in 0.1 M phosphate buffer. Thus the COMT activity appeared to be related to the soluble fraction of the cytoplasm from brain tissue.

The COMT activity was determined in various regions of brain, as well as the salivary gland and the liver, all the tissues being obtained from the rat. The order of enzyme activity obtained for the three different tissues was: liver > salivary gland > brain, which is in accordance with the literature No comparison can be made with previously published studies and our results with regard to the regional distribution of COMT activity in rat brain A comparison with other studies of the absolute values for the enzyme activity is difficult since both substrates and preparations of enzyme vary A radioactive method using ^{3}H-SAM (Uretsky & Iversen 1970) appeared to have comparable experimental conditions The values for COMT activity using the radiochemical method are about twice as high as those obtained by the present method. It is considered possible that different commercial sources of the SAM preparations contributed significantly to these differences

REFERENCES

Assicot, M & C. Bohuon: Life Sci 1969, 8 93-100
Uretsky, V J & L L. Iversen: J Neurochem 1970 17 269-278

Odd Brørs and Sten Jacobsen (*Institute of Pharmacology, University of Oslo, Norway*): IONIC BASIS OF BRADYKININ ACTION ON THE RAT UTERUS

Bradykinin and angiotensin are said to act on the cell by changing the flux of ions across the membrane According to recent theories the ions cross cell membranes by ion exchanger mechanisms. On the surface of most cells there seems to be an excess of fixed negatively charged sites, conferring to a cation exchanger system.

The action of bradykinin on the smooth muscle could involve a binding of the polypeptide molecules to such negative charged sites on the smooth muscle and be influenced by hydrogen ion-concentration and pKa values of specific groups on the smooth muscle and the bradykinin molecule.

The problem whether the polypeptide molecule and the receptor (functional site) on the rat uterus interact in the ionized or the unionized form was examined on the isolated rat uterus preparation.

The responses to different concentrations of bradykinin at different pH levels (6 to 9) were determined, and concentration-response curves plotted according to Lineweaver Burk. The curves obtained are straight and have a common point of intersection with the ordinate, suggesting a competition between bradykinin molecules and hydrogen ions for a common functional site on the rat uterus Experimental isoboles representing the relation, the pH and concentration of bradykinin giving 50 % of maximum contraction, have a bend at a pH of about 8 3, suggesting a pKa of 8.3 of the functional site on the rat uterus

The influence of drugs (chlorpromazine and diethylstilboesterol) and chemical agents (mercurichloride, parachloromercuri-benzoic acid and dithiothreitol) on the effect of bradykinin on the rat uterus was examined. Based on the results obtained and observations from bradykinin analogues it is postulated that guanidine groups on the bradykinin molecule react with sulphydryl groups on the isolated rat uterus

S. Carlsson, O. Eriksson, A. Sundwall, K. Uthne and J. Vessman (Research Department of the KABI Group, Stockholm, Sweden): THE METABOLIC DISPOSITION OF TERODILINE (BICOR[R]).

The absorption, distribution, metabolism and excretion of ^{14}C-labelled terodiline were studied in mouse, rat and dog. Unchanged terodiline was determined by gas chromatography as described by Vessman & Strömberg (1969). The drug was very rapidly absorbed after oral administration and most of the radioactivity excreted in the urine was in the form of metabolites. The amount of radioactivity and unchanged drug excreted in the urine in 6 hours is shown in the table.

	Mouse		Rat		Dog	
	% ^{14}C	% unchanged	% ^{14}C	% unchanged	% ^{14}C	% unchanged
i.v.	67	7.6	21		30	< 1
p.o.	66	6.2	14		35	< 1

In the dog 20 - 40 % of the intravenously injected radioactivity were found in the faeces indicating a very extensive biliary excretion. Less than 1 % was present as the unchanged drug.

The metabolites in the urine were separated by paper chromatography and/or paper high voltage electrophoresis. In mouse urine at least five major metabolites could be separated, two of which were shown to be glucuronides (Rf 0.04, 0.19, 0.37 and 0.84). Marked species differences were noticed, since in the rat urine only one major metabolite was found (Rf 0.20). In dog urine metabolites with the Rf 0.08, 0.16, 0.78 were found following intravenous administration. After oral administration an additional major peak with the Rf 0.39 occurred. The compounds with Rf 0.17 - 20, 0.37 - 39 are presumably glucuronides.

p-hydroxyterodiline was synthesized and one of the major metabolites was found to be indistinguishable from the synthetic compound in the paper chromatographic (Rf 0.84) and electrophoretic systems used

The half-life of terodiline in dog plasma was found to be 2 - 3 hours which is much shorter than in man where the corresponding figure was found to be 3 days. No difference in antipyrine half-life could be demonstrated after 3 weeks daily administration (dogs). Preliminary experiments show that terodiline is bound to more than 80 % to dog plasma.

REFERENCE

Vessman, J. & S. Strömberg: Acta pharm. Suecica 1969, 6, 505-518

Rune Dahlquist and Bertil Diamant (Department of Pharmacology, Karolinska Institutet Stockholm Sweden) FURTHER OBSERVATIONS ON ATP INDUCED HISTAMINE RELEASE FROM RAT MAST CELLS

Histamine release from isolated rat mast cells is induced by ATP ($>10^{-5}$ M) in the presence of Ca^{++} In the presence of Mg^{++} the release is inhibited (Diamant & Krüger 1967)

ATP induced histamine release showed a narrow pH optimum around 7 0 Decreasing the pH to 6 3 or increasing it to 7 8 inhibited the release almost completely Histamine release in contrast only little reduced by a five- hydrogen ion concentration from pH 7 at this pH

ATP did not induce any histamine release at 0 °C but was as effective as releasing agent at room temperature as at 37 °C At temperatures above 40 °C the reduction in ATP-induced histamine release was markedly greater than for compound 48/80 induced release

Of the triphosphonucleotides tested only ATP was effective as histamine releasing agent In addition CTP GTP ITP TTP or UTP did not influence the amine releasing action of ATP when present simultaneously

With 0 1 mM to 1 mM of Ca^{++} ATP-induced histamine release was maintained fairly constant at an optimal level Higher concentrations of Ca^{++} than 1 mM were inhibitory and concentrations below 0 1 mM were not sufficient to give optimal release with ATP

The inhibitory action of Mg^{++} was shown to be complete (i e no release occurred) with Mg^{++} between 0 5 and 1 mM irrespectively of the concentration of Ca^{++} indicating an inhibitory mechanism of Mg^{++} not competitive with Ca^{++}

When mast cells were preincubated with ATP (3×10^{-5} M) for 2 min prior to the addition of Ca^{++} (1 mM) the percentage histamine released was considerably higher than when ATP and Ca^{++} were added together to the cells Furthermore preincubation of the cells a few minutes prior to the addition of Ca^{++} (1 mM) with an ATP concentration (10^{-5} M) that did not cause any histamine release when added to the cells together with Ca^{++} resulted in a marked release When the preincubation time with ATP was extended to 10 min before the addition of Ca^{++} no histamine release occurred however

The time course for ATP induced histamine release was slow as compared to the release caused by degranulating agents like compound 48/80 A lag period was observed before histamine release started The length of the lag period shortened and the initial velocity of the release as well as the final amount of amine released increased when the ATP-concentration was increased from 2×10^{-5} M to 5×10^{-5} M Similarly pretreatment of the cells for 2 min with ATP (3×10^{-5} M) before the addition of Ca^{++} (1 mM) resulted in a shorter lag period higher release velocity and a higher percentage histamine released as compared to when ATP and Ca^{++} were added together to the cells

REFERENCE

Diamant B & P G Krüger Acta physiol scand 1967 1 291 302

S. Carlsson, O. Eriksson, A. Sundwall, K. Uthne and J. Vessman (Research Department of the KABI Group, Stockholm, Sweden): THE METABOLIC DISPOSITION OF TERODILINE (BICOR[R]).

The absorption, distribution, metabolism and excretion of ^{14}C-labelled terodiline were studied in mouse, rat and dog. Unchanged terodiline was determined by gas chromatography as described by Vessman & Strömberg (1969). The drug was very rapidly absorbed after oral administration and most of the radioactivity excreted in the urine was in the form of metabolites. The amount of radioactivity and unchanged drug excreted in the urine in 6 hours is shown in the table.

	Mouse		Rat		Dog	
	% ^{14}C	% unchanged	% ^{14}C	% unchanged	% ^{14}C	% unchanged
i.v.	67	7.6	21		30	< 1
p.o.	66	6.2	14		35	< 1

In the dog 20 - 40 % of the intravenously injected radioactivity were found in the faeces indicating a very extensive biliary excretion. Less than 1 % was present as the unchanged drug.

The metabolites in the urine were separated by paper chromatography and/or paper high voltage electrophoresis. In mouse urine at least five major metabolites could be separated, two of which were shown to be glucuronides (Rf 0.04, 0.19, 0.37 and 0.84). Marked species differences were noticed, since in the rat urine only one major metabolite was found (Rf 0.20). In dog urine metabolites with the Rf 0.08, 0.16, 0.78 were found following intravenous administration. After oral administration an additional major peak with the Rf 0.39 occurred. The compounds with Rf 0 17 - 20, 0.37 - 39 are presumably glucuronides.

p-hydroxyterodiline was synthesized and one of the major metabolites was found to be indistinguishable from the synthetic compound in the paper chromatographic (Rf 0.84) and electrophoretic systems used.

The half-life of terodiline in dog plasma was found to be 2 - 3 hours which is much shorter than in man where the corresponding figure was found to be 3 days. No difference in antipyrine half-life could be demonstrated after 3 weeks daily administration (dogs). Preliminary experiments show that terodiline is bound to more than 80 % to dog plasma

REFERENCE

Vessman, J. & S Strömberg: Acta pharm. Suecica 1969, 6 505-518

Gilles Fillion and Salvador Lluch (Department of Pharmacology, Karolinska Institutet Stockholm Sweden) RELEASE OF NORADRENALINE FROM THE DOG HEART IN SITU FOLLOWING ADMINISTRATION OF 5 HYDROXY-TRYPTAMINE (5 HT)

The hypothesis that the stimulatory effects of serotonin (5-HT) on the dog heart are mediated by catecholamines has been proposed on the basis of pharmacological evidence In the present work performed on anaesthetized open chest dogs the release of noradrenaline from the heart following administration of 5 HT was investigated using biochemical methods

After intravenous injections of 5 HT (5 to 100 μg/kg) an increase in noradrenaline content of the coronary sinus blood was detected using a fluorimetric assay In order to avoid the interference of possible simultaneous release of noradrenaline from the periphery 5 HT (1 to 100 μg) was injected into the coronary circulation after the heart had been labelled with ^{3}H-noradrenaline The release of ^{3}H noradrenaline into the coronary venous blood was enhanced after 5 HT but no significant change in the vena cava blood occurred

In further experiments using hearts labelled with ^{3}H-5 hydroxytryptamine stimulation of the cardioaccelerator nerves released radioactivity into the coronary venous blood

It is concluded that the release of noradrenaline from the heart by 5-HT strongly supports the hypothesis that the stimulatory effect of 5-HT *is a* catecholamine-mediated mechanism Furthermore on the basis of the experiments with ^{3}H 5 hydroxytryptamine labelled hearts an uptake *of* 5-HT by the nerve endings may occur

Bertil Diamant and Rune Dahlquist (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): THE INHIBITORY ACTION OF ADENOSINE-5'-TRIPHOSPHATE (ATP) ON HISTAMINE RELEASE AND MAST CELL DEGRANULATION INDUCED BY COMPOUND 48/80

Histamine release from rat mast cells induced by compound 48/80 differs from that induced by ATP in regard to morphological changes energy requirement, divalent cation dependence and time course (Diamant & Krüger 1967; Diamant 1969) The two agents thus seem to initiate histamine release by two different cellular mechanisms The present investigation concerns the interaction between the two releasing agents on the mast cells with special regard to the influence of ATP on 48/80-induced mast cell degranulation and histamine release.

When isolated rat mast cells were exposed to a mixture of 48/80 and ATP (>10 μ M) mast cell degranulation and histamine release were inhibited as compared to the effect of 48/80 alone Inhibition of the effect of 48/80 also occurred with ATP at concentrations between 2 and 10 μ M provided the cells were preincubated with ATP prior to the addition of 48/80 With ATP (3 - 7μ M), the inhibition spontaneously disappeared, when the cells were exposed to prolonged incubation with ATP. With increasing concentrations of ATP the inhibition was maintained in spite of prolonged incubation prior to the addition of 48/80 In addition, the inhibition was maintained after washing of the ATP-treated cells prior to exposure to 48 80 The inhibitory effect of ATP was counteracted by the presence of Ca^{++} and Mg^{++} in the incubation medium during the time the cells were exposed to ATP The spontaneous restoration of the sensitivity of ATP-treated cells towards the action of 48/80 by prolonged incubation varied in different experiments It was enhanced, however, by the presence of glucose The spontaneous restoration with time of the sensitivity of mast cells towards 48/80, the enhancing effect of glucose on the restoration as well as the protecting action of Mg^{++} and Ca^{++} on the inhibitory action of ATP were all effects found to be counteracted by the presence of EDTA

The observed inhibition by ATP on the action of 48/80 occurred under the same experimental conditions as observed to cause increased sodium content and decreased potassium content of the cells. It is therefore suggested that ATP by itself induced changes of the cell membrane which unabled 48/80 to exert its action It is evident that this effect of ATP was exerted at concentrations which did not release histamine from the cells under suitable conditions (e g in the presence of Ca^{++}) and that the histamine releasing process induced by ATP did not parallel the experimental conditions necessary for ATP to inhibit the action of 48 80

REFERENCES

Diamant, B Int Arch Allergy 1969 36 3 21
Diamant B & P G Krüger Acta physiol scand 1967 71 291 302

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TRYPTAMINE (5-HT)

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REFERENCES

Diamant B Int Arch Allergy 1969 36 3 21
Diamant B & P G. Kruger Acta physiol scand 1967 71 291 302

H H Frey and M P Magnussen (Department of Pharmacology Leo Pharmaceutical Prod Ballerup Denmark and Department of Pharmacology and Toxicology School of Veterinary Medicine Free University Berlin Germany) CONTRIBUTION TO THE ANTICONVULSANT EFFECT OF PHENOBARBITAL

In mice the ED 50 in the maximal electroshock seizure test was determined at different intervals after the oral administration of phenobarbital The maximal effect was after 2 4 hours (ED 50 13 15 mg/kg) then the ED 50 rose to values of 20 mg/kg after 6 and 8 hours 31 mg/kg after 12 36 mg/kg after 16 39 mg/kg after 20 and 46 mg/kg after 24 hours When the phenobarbital concentration in serum and brain was determined after application of the respective ED 50s and at the respective points of time average levels of 18 μ g/ml in serum and 20 μ g/g in brain were found from the second to the eighth hour Beginning from 12 hours both concentrations showed a falling tendency so that after 24 hours only 10 5 μ g/ml were found in serum and 9 1 g/g in brain Thus at the later points of time, the mice were protected against electroconvulsions by definitely lower phenobarbital concentrations than during the first 8 hours In order to explain this change in sensitivity to the anticonvulsant drug action being just opposite to the wellknown phenomena of acute tolerance ' and 'tolerance the content and turnover of several biogenic amines and related agents in the brain of mice were studied The turnover of noradrenaline was lowered after 2 and 4 hours and had normalized at 20 and 24 hours whereas the turnover of 5-HT was enhanced at the short time intervals and definitely lowered after 20 and 24 hours No changes were found in the central levels of noradrenaline dopamine 5 HT γ amino butyric acid and acetylcholine Thus an imbalance between the turnover of noradrenaline and 5 HT may be the explanation for the phenomenon observed

Bertil B. Fredholm (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): METABOLIC AND CIRCULATORY EFFECTS OF THEOPHYLLINE IN CANINE SUBCUTANEOUS ADIPOSE TISSUE.

Methyl xanthines, including theophylline, are capable of inhibiting the enzymatic breakdown of cyclic AMP. For this reason theophylline has been widely used to assess the role of cyclic AMP in the regulation of many physiological processes.

In the present study theophylline-1-aminopropanol was infused in graded amounts (10^{-5} - 10^{-2} M in blood) to canine subcutaneous adipose tissue. Maximal vasodilatation was produced by about 3×10^{-4} M theophylline. The effect was immediate. An increased tissue volume, indicating a venodilatation, a net outward filtration of fluid as well as 2 to 4 fold increase in capillary filtration coefficient were also seen.

The rates of FFA and glycerol release were increased. This effect developed gradually over a period of 4 to 15 min. A maximal effect, 4 - 10 μmoles/min/100 g, was produced by concentrations above 4×10^{-3} M. The FFA release rate increased more than did the glycerol release rate, indicating a decreased re-esterification ratio. The effect of sympathetic nerve stimulation was potentiated by lower (less than 10^{-3} M) concentrations of theophylline, but nerve stimulation did not affect the maximal release rates obtained with high concentrations of theophylline Glucose and lactate uptakes were not significantly affected, whereas the pyruvate production increased, indicating an increased glycogenolysis.

The results agree with the opinion that cyclic AMP is continuously and rapidly synthetized and broken down in unstimulated adipose tissue and that nerve stimulation can further increase the formation of cyclic AMP, thereby increasing the breakdown of the lipid and carbohydrate stores

MULATED RAT ATRIA

It has previously been shown that phenothiazine derivatives affect the excitability and contractility of isolated cardiac tissue in the same way as quinidine and local anaesthetics In an attempt to determine the ionic mechanisms underlying the effect of these drugs the influence of phenothiazines on ^{42}K efflux from isolated rat atria has been studied The atria were excised in ether anaesthesia tied to a holder and submerged in a modified Ringer solution where the KCl had been replaced by ^{42}KCl After one hour the muscles were transferred to a glass chamber which allowed electrical stimulation of the tissue and tension recording The radioactive ^{42}K was washed out of the muscles either by Ringer solution or by Ringer solution to which promazine or thioridazine (5 10^{-6} - 10^{-5} M) had been added The washout was collected in two minute samples and the radioactivity of each sample was determined

Both promazine and thioridazine reduced the ^{42}K efflux promazine being the most potent when compared on a molar basis This may explain why the phenothiazines cause a prolongation of the repolarization phase of the act on potentials in rat atria (Landmark unpublished)

The lowest concentration of drug required to decrease the ^{42}K efflux gave no significant reduction in contractility whereas higher dosages reduced the tension developed during the contractions This is in accordance with an earlier observation that the lowest concentrations required to alter the electrocardiogram of isolated perfused rat hearts cause no significant reduction in cardiac output (Landmark et al 1969)

REFERENCE

Landmark K A Glomstein & I Øye Acta pharmacol et toxicol 1969, 27 173 182

Marianne Frisk-Holmberg (Department of Pharmacology Karolinska Institutet, Stockholm, Sweden) FURTHER STUDIES ON THE MECHANISM OF CHLORPROMAZINE (CPZ) INDUCED HISTAMINE RELEASE FROM RAT MAST CELLS CHANGES IN THE CELLULAR ION COMPOSITION

It is known that CPZ releases histamine in vivo and in vitro from various tissues in several species It has recently been shown (Frisk-Holmberg 1969) that CPZ in concentrations 2 8 10^{-6} - 5 6 10^{-5} M released 5 - 70 % of rat mast cell histamine content in vitro The histamine release did not seem to be accompanied by degranulation Decreased ionization of the drug enhanced and tetrodotoxin in concentrations 10^{-6} - 10^{-4} M reduced histamine release in agreement with a membrane action of CPZ

As it is known that CPZ changes membrane permeability the intracellular ion composition of the mast cells after exposure to CPZ was studied Sodium and potassium were measured in a flame-photometer Increasing CPZ concentrations 2 8 - 8 4 10^{-5} M increased cellular sodium content and decreased potassium content Above CPZ 5 10^{-5} M potassium efflux was accompanied by an efflux of a cytoplasmatic enzyme lacticdehydrogenase (LDH) (measured according to Lowry 1961)

It is concluded that CPZ changes the mast cell Na^+ and K^+ concentrations This might be due to alterations in mast cell membranes by CPZ Increased Na^+ content is theoretically a state in which histamine may be released from granular binding sites Concentrations of CPZ releasing more than 60 % of total histamine content lead to efflux of LDH indicating a radical change of the cell membrane

REFERENCES

Frisk-Holmberg, M Acta physiol scand 1969 Suppl 330 63
Lowry, O H , J V Passonweau D W Schulz & M K Rock J biol Chem 1961 236 2746-2755

. University of Copenhagen, Den-

BY SLICES OF MOUSE KIDNEY

.. _ . ROPINE

The stable cholinergic agent decamethonium is concentrated in the mouse kidney in vivo (Broen Christensen & Holm 1969) Slices of mouse kidney accumulate decamethonium and the uptake is inhibited by the pharmacological antagonist d-tubocurarine (Holm & Broen Christensen 1969) The present study was made to investigate the uptake of another stable cholinergic agent, carbamoylcholine, by mouse kidney slices with particular reference to the inhibitory effect of the pharmacological antagonist atropine.

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Carbamoylcholine (2×10^{-6} M) was taken up rapidly by kidney slices incubated in an atmosphere of oxygen-carbondioxide (95:5) Thus, within 1/2 hour of incubation a steady state was obtained in the distribution of carbamoylcholine with a S/M ratio around 10 The S/M ratio decreased when raising the carbamoylcholine concentration The radioactive material extracted corresponded chromatographically to authentic carbamoylcholine In an atmosphere of nitrogen-carbondioxide (95:5) the S/M ratio reached a constant value of about 1 within 1/2 hour (2×10^{-6} M carbamoylcholine) and did not decrease when raising the carbamoylcholine concentration The uptake (S/M ratio) of carbamoylcholine was markedly inhibited in the presence of metabolic inhibitors When the carbamoylcholine concentration was 2×10^{-6} M atropine (2×10^{-6} M) reduced the S/M ratio by 40 %, ten and hundredfold higher concentrations of atropine reduced the S/M ratio by 75 and 90 % respectively

The results suggest that carbamoylcholine is taken up against a concentration gradient by a partly energy dependent process, which follows saturation kinetics The uptake consists of an atropinesensitive part and a part much less sensitive to the inhibitor

REFERENCES

Broen Christensen, Chr & J Holm, Acta pharmacol et toxicol, 1969, 27, 17-26

Holm J & Chr Broen Christensen, Acta physiol. scand 1969, 77 (Suppl. 330) 69

M. Henning and A. Rubenson (Department of Pharmacology, University of Göteborg, Sweden): EFFECTS OF L-DOPA AND STRUCTURALLY RELATED COMPOUNDS ON BLOOD PRESSURE

Mean arterial blood pressure (MAP) was recorded in conscious normotensive rats through in-dwelling arterial catheters (Henning 1969) Drugs were given intraperitoneally.

L-dopa alone always produced a hypertensive response. After pretreatment with a potent inhibitor of both central and peripheral dopa decarboxylase (seryl-2,3,4-trihydroxybenzyl-hydrazine, Ro 4-4602, 400 + 200 mg/kg), L-dopa up to 200 mg/kg had no effect on MAP. However, after selective inhibition of peripheral decarboxylase by α-hydrazine-α-methyl-β-(3,4-dihydroxyphenyl) propionic acid (MK 185, 100 mg/kg), L-dopa 200 mg/kg produced a significant lowering of MAP. Thus, decarboxylation of L-dopa in both central and peripheral tissues gave a hypertensive response; decarboxylation in the central nervous system resulted in a decrease in MAP This central hypotensive effect of L-dopa was abolished by an inhibitor of dopamine β-hydroxylase, bis-(4-methyl-1-homopiperazinyl-thiocarbonyl)-disulfide (FLA-63, 40 mg/kg) A central dopamine receptor blocking agent (spiroperidol, 0 1 mg/kg) had no effect Previous work has established that the antihypertensive effect of L-α-methyl-dopa (MD) is abolished after pretreatment with Ro 4-4602 but not with MK 485, indicating that MD must be decarboxylated in the central nervous system to exert its hypotensive effect (Henning 1968, 1969) We now report that this effect was abolished by pretretment with a β-hydroxylase inhibitor (FLA-63 25 mg/kg before MD 200 mg/kg), which in parallel experiments prevented the formation of α-methylnoradrenaline from MD.

DL-meta-tyrosine (MT) has been studied in analogy with L-dopa. MT 400 mg/kg increased MAP when given alone but had no effect after Ro 4-4602 After MK 485 MT 400 mg/kg lowered MAP significantly with a more rapid onset and shorter duration than L-dopa The hypotensive response was blocked by FLA-63 but not by spiroperidol

DL-α-methyl-metatyrosine (MMT) has previously been shown to lack hypotensive effect in conscious rats (see Henning 1969) We have now observed that this is still the case after pretreatment with MK 485

Thus, L-dopa and MD both produce hypotensive responses via central actions of their amine metabolites and noradrenergic mechanisms seem to be involved Studies from this laboratory (Andén et al 1970) have shown that L-dopa and MD but not MMT are capable of stimulating central noradrenaline (NA) receptors through their metabolites Interestingly the antihypertensive drug, clonidine (Catapresan[R]) also stimulates central NA receptors

REFERENCES

Andén N -E , S G Butcher & J Engel Acta pharmacol et toxicol 1970, 28, Suppl. 1
Henning, M Brit J Pharmacol 1968 34 233P-234P
Henning M : Acta physiol. scand 1969 Suppl 322

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The positive inotropic effects of catecholamines have been postulated to result from an increase in the intracellular level of cyclic 3 5 -AMP

cyclic 3 5 -AMP nor d butyryl cyclic 3 5 -AMP in the perfused rat heart Kukovetz (1968) reported a positive inotropic effect and an activation of the phosphorylase system when dibutyryl cyclic 3 5 AMP was administered to non working perfused hearts (Langendorff preparation)

In the present study rat hearts were perfused anterogradely as described by Morgan and co workers (1965) which makes it possible for the heart to perform a real mechanical work in vitro Hearts from rats weighing 80 g were perfused at 37 °C with Krebs bicarbonate buffer containing glucose as substrate Dibutyryl cyclic 3 5 AMP caused a concentration-dependent increase in peak systolic pressure coronary flow and cardiac output the threshold concentration being 10^{-4} M In contrast to the rapid effects of adrenaline seen within 10 sec there was a time lag of 3-5 min before the effects of d butyryl cyclic 3 5 AMP were demonstrated Glycogen content was markedly decreased after a perfusion period of 60 min with dibutyryl cyclic 3 5 AMP Although propranolol completely blocked the inotropic and glycogenolytic effects of adrenaline it did not influence the effects of d butyryl cyclic 3 5 AMP

The sympatomimetic effect of cyclic 3 5 AMP in this study is probably due to the fact that the dibutyryl derivate of cyclic 3 5 -AMP enters the cells more readily and is resistent to enzymatic degradation by phosphodiesterase Also compared to previous studies younger rats have been used and the cell membranes of these rats may have better permeability properties which facilitates the entrance of cyclic 3 5 AMP to the cell interior

REFERENCES

Kukovetz W R Arch Pharm exp Path 1968, 260 163 164
Morgan H E J R Neely R E Wood C Liebecq H Liebermeister & C R Park Fed Proc 1965 24 1040 1045
Robison G A R W Butcher I Oye H E Morgan & E W Sutherland Molec Pharmacol 1965 1 168 177
Oye I The mode of action of adrenaline in the isolated rat heart Universitetsforlaget Oslo 1967 9 55

Ivar Hørven and Per Syrdalen (Oslo University Eye Department, Rikshospitalet, Norway): CORNEAL INDENTATION PULSE DURING GENERAL AND RETROBULBAR ANAESTHESIA.

Hydrodynamically the vascular bed of the eye may be divided into a non-pulsatile and a pulsatile part. Through the non-pulsatile part the blood flows at a constant volume per time unit in diastole and systole, while the pulsatile part receives blood only in systole. This excess of blood entering the eye in systole will induce a rise in intraocular pressure which is dependent upon the intraocular pressure level and ocular rigidity, and which may be recorded in microns of plunger movements by use of dynamic tonometry (Hørven 1968). The amplitudes in microns may be converted into corresponding changes in intraocular volume or pressure by converting tables presented elsewhere (Hørven 1970).

If the vascular bed of the eye increases (choroidal melanoma or hemangioma) or if the pulsatile bolus of blood increases (bradycardia), a corresponding increase may be observed in the corneal indentation pulse If the vascular bed of the eye is decreased (tapetoretinal degeneration) or a decrease exists in the pulsatile bolus of blood reaching the eye (tachycardia, giant cell arteritis, carotid occlusive disease) the corneal indentation pulse will show a corresponding decrease.

In the present study the corneal indentation pulse was recorded before and during general anaesthesia (ether halothane) and before and 10 minutes after retrobulbar anaesthesia with results as follows (N = number of eyes):

		General anaesthesia	Retrobulbar injection (4 5 ml):			
			Xylocain 1 %	Exadrin	Xylocain -Exadrin 1 %	Saline 0 9 %
		N = 39	N = 20	N = 3	N = 20	N = 3
Before	μ	31 44	29 35	24 67	27 15	32 33
	σ	12 29	10 14	10 26	13 11	9 07
During	μ	10 43	15 65	13 33	8 40	32 33
	σ	7 38	8 66	7 09	7 26	8 14
% = During · 100 / Before		33	53	54	31	100

A striking decrease of similar order of magnitude was initiated by retrobulbar injection of either Xylocain or Exadrin Evidence is offered suggesting that the two drugs act through different trigger mechanisms in their corneal indentation pulse decreasing capacity as a combined effect is obtained when the two drugs are injected simultaneously The amplitude decrease was not related to any changes in general blood pressure intraocular pressure or ocular rigidity, and its nature is obscure It is obvious, however, that experimental studies on ocular hydrodynamics and blood supply performed during general of retrobulbar anaesthesia will not reflect the normal state of non-anaesthetized subjects

REFERENCES

Hørven I.: Acta ophthal , Kbh 1968, 46 1213-1221
Hørven I · Acta ophthal , Kbh 1970 48 23-38

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REFERENCES

Kukovetz W R Arch Pharm exp Path 1968 260 163-164
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Robison G A R W Butcher I Oye H E Morgan & E W Sutherland Molec Pharmacol 1965 1 168 177
Oye I The mode of act on of adrenaline in the isolated rat heart Universitetsforlaget Oslo 1967 9 55

Jens Aas Jansen, E Hvidberg and H. H Lausen (Department of Pharmacology, University of Copenhagen, Denmark): THE PROTEIN BINDING OF PLASMA CORTISOL AFTER THERAPEUTIC DOSES OF INDOMETHACIN

The aim of this investigation has been to establish if indomethacin in therapeutic concentrations interferes with the protein binding of plasma cortisol. In trying to solve this problem also plasma concentrations of indomethacin during ordinary medication had to be determined

Available methods for indomethacin determinations in plasma seemed insufficient An existing spectrophotofluorometric method was modified resulting in a greatly improved sensitivity (50 ng in half a millilitre) and accuracy. This improvement was obtained by measuring the fluorescence of indomethacin in a phosphate buffer with pH 11.6 instead of 0 1 N-NaOH. Concerning specificity salicylic acid was the only problem

Two series of investigations on humans were conducted.

The mean concentration of indomethacin was 0.8 μg/ml (range 0 1-1.5) in plasma from a number of patients treated with indomethacin capsules for more than two days One blood sample was drawn 45-150 minutes after the last intake of 25 mg of indomethacin.

Blood samples were taken at intervals of twenty minutes from four healthy male volunteers after oral intake of 75 or 100 mg of indomethacin (1 1-1 25 mg/kg) Three of these showed the highest concentration (6, 16 and 18 μ g/ml) 40-50 minutes after intake The fourth volunteer did not reach the maximum (5 μ g/ml) until 4 hours after intake The decay of indomethacin in plasma is not rectilinear in a semi-logarithmic plot as the slope decreases gradually Protein binding of the drug may account for this phenomenon Initially the slope corresponds to a half-life of 70-80 minutes The protein binding of plasma cortisol was increased compared to normal values in three of the volunteers 140 minutes after the intake of indomethacin

In vitro experiments (2, 5 or 20 μ g/ml indomethacin to plasma) did not alter the protein binding of plasma cortisol in the actual sample

In conclusion therapeutic concentrations of indomethacin may interfere with the protein binding of plasma cortisol but seemingly not through a direct action. This is in accordance with previous experiments

Jens Jensen Holm (Department of Biophysics University of Copenhagen, Denmark) TRIS (HYDROXYMETHYL) AMINOMETHANE AS AN ACTIVATOR OF ACETYLCHOLINESTERASE

Tris is a commonly used buffer in cholinesterase histochemistry (Eränkö et al 1967) although Pavlič (1967) using the Warburg manometric technique has described an inhibitory effect of Tris on both acetylcholinesterase and cholinesterase However when buffer solutions without Ca^{++} and Mg^{++} were used Pavlič found that Tris activated the two enzymes

In the present study a titrimetric method (Jensen Holm 1961) was used for determination of acetylcholinesterase activity The method employs human ghosts in normal saline at 38 °C pH 7 40 or 6 15 and acetylcholine iodide as substrate at concentrations varied between 6×10^{-6} and 10^{-2} M

It was found that the addition of Tris (chloride or acetate) at final concentrations from 0 3 to 5 *mM caused a pronounced increase of the enzyme activity*, provided that the concentration of acetylcholine was above 10^{-4} M Below this concentration of acetylcholine no effect of Tris was obtained The results were identical in the presence and absence of Ca^{++} and Mg^{++} The rate of spontaneous hydrolysis of acetylcholine was not influenced by Tris

As the relative ability of Tris to activate the enzyme was much more pronounced at pH 7 40 than at 6 15 it is likely that the activation mechanism is related to its non ionized form

REFERENCES

Eränkö O G B Koelle & L Räisänen J Histochem Cytochem 1967 15 674 679

Jensen Holm J Acta pharmacol et toxicol 1961 18 379-397

Pavlic M Biochim biophys Acta 1967 139 133 137

Jens Jensen-Holm and Per Juul (Institute of Pharmacology, University of Copenhagen, Denmark): ULTRASTRUCTURE OF RAT SYMPATHETIC GANGLIA FOLLOWING GUANETHIDINE.

Prolonged administration of guanethidine intraperitoneally to rats at doses from 10 to 20 mg/kg induced an enlargement of the superior cervical ganglia and a decrease in the activities of specific and non-specific cholinesterases from both the cytoplasm of the nerve cells and from the nerve fibres. Further a considerable, but reversible chromatolysis of the nerve cells and an infiltration of small cells were found (Jensen-Holm & Juul 1970a, 1970b).

In the present investigation the morphological changes of the rat sympathetic ganglia have been studied by means of electron microscopy. Profound ultrastructural changes consisting of a marked irregular swelling of the nerve cell mitochondria with partial loss of cristae are observed. Further an increase in the number of dense bodies is seen. The changes are visible after 10 mg/kg for 14 days and further developed after 20 and 40 mg/kg for 14 days. The changes are reversible - partial normalization having occurred within 3 weeks following discontinuation. Only minor changes in the pre- and postganglionic axons are observed. Similar ultrastructural changes are not seen in liver and salivary glands. Although the changes induced by guanethidine in some ways resemble those resulting from postganglionic axotomy they are much more pronounced. Malmquist & Oates (1968) demonstrated that guanethidine partially inhibited the oxidative phosphorylation of rat liver mitochondria in vitro. As guanethidine is assumed to be cumulated in sympathetic neurons, the mechanism of action of long term administration may be an interference with normal mitochondrial function.

REFERENCES

Jensen-Holm, J. & P. Juul: Acta pharmacol. et toxicol. 1970a, 28. In press
Jensen-Holm, J. & P. Juul: Acta pharmacol et toxicol. 1970b, 28. In press
Malmquist, J. & J. A Oates: Biochem. Pharmacol. 1968, 17, 1845-1854.

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LAPSE CINEMICROGRAPHIC STUDY

We have found that the commonly used herbicide 2 4-dichlorophenoxyacetic acid (2 4 D) inhibits the growth of mouse fibroblasts (Earle s L cells) in monolayer cultures The inhibition was accompanied by a transient accumulation of lipid containing vacuoles in cytoplasm Upon removal of 2 4-D from the cultures the growth was resumed

For time lapse cinemicrography L cells were grown as monolayer cultures in Sykes and Moore chamber in Eagle s Minimum Essential Medium supplemented with 10 % calf serum The cultures were photographed under phase contrast with a Reichert inverted microscope at 2 frames/min by using a Beaulieu camera and 16 mm Kodachrome II A film The cells were incubated for 24 hours at 37 °C after transferring to the chamber Test medium with 500 μg/ml was then added and the recording was started During 33 hours no cell divisions were seen and some accumulation of vacuoles occurred Upon the subsequent perfundation of the chamber with control medium a rapid resumption of growth took place There was no evidence as to a synchronizing effect of 2 4 D Within 22 hours in control medium the cells were free from vacuoles No obvious effect of 2 4-D on pinocytosis and the undulating activity of cell membranes were seen The film will be presented before the meeting

John Kristoffersen and Per Løkken (Department of Pharmacology and Toxicology, Veterinary College of Norway, and Institute of Pharmacology, University of Oslo, Norway): ^{51}Cr-EDTA AS AN ALTERNATIVE TO PHENOL RED IN A RAT TEST MEAL TECHNIQUE.

In a test meal technique introduced for the study of gastric secretion in rats, Thornton & Clifton (1959) selected phenol red as a reference substance. Experiences with ^{51}Cr-EDTA as a gastro-intestinal reference substance in rats (Løkken & Søgnen 1967; Løkken 1970) suggested that this substance may offer advantages and may improve the test meal method.

In fasted rats test meals containing phenol red and/or ^{51}Cr-EDTA were instilled intragastrically. After histamine stimulation the gastric contents were aspirated and rinsed out with water after 45 minutes. pH-measurements, titrations of HCl, spectrophotometric phenol red determinations and ^{51}Cr-activity assays were performed on aliquots of the gastric contents plus washout. The recoveries of the reference substances were used to calculate the volume of gastric contents, the amounts of HCl passed through the pylorus and, thereby allowing calculation of the total quantity of HCl secreted.

When the two reference substances were given to different groups of rats, the mean recovery of phenol red (37.0 %) was significantly lower than the corresponding value with ^{51}Cr-EDTA (48.7 %). Consequently, when calculations were based on phenol red as a reference substance, significantly larger volumes of fluids and higher amounts of HCl were estimated to have passed through the pylorus. In a paired comparison where the compounds were given in the same test meal similar results were obtained.

Absorption and binding of phenol red to the gastric mucosa are discussed as possible explanations for the discrepancy in the recoveries of ^{51}Cr-EDTA and phenol red from the gastric aspirates.

Due to its stability, inertness, more complete recovery and the simple analytical procedure, ^{51}Cr-EDTA in the present test meal method seems to meet the requirements of a reference substance better than phenol red

REFERENCES

Løkken, P : Studies on ^{51}Cr-EDTA and its evaluation as a reference substance in gastro-intestinal research. (Thesis) Universitetsforlaget Oslo, 1970.

Løkken, P. & E. Søgnen: Acta pharmacol. et toxicol. 1967, 25 (Suppl 4), 39.

Thornton, G. H. M. & J A. Clifton: Amer. J Physiol 1959, 197 263-268

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IA CAUSED BY PHENOTHIA

Phenotl iazine derivatives cause a wide range of electrocardiographic (ECG) disturbances in patients The most common changes are prolongation of the QT interval notching or lowering of the T wave alterations of the QRS complex and varying degrees of atrioventricular dissociation (review Landmark 1970) Similar changes in the ECG have been found of isolated perfused rat hearts after addition of several phenothiazines (Landmark et al 1969 Langslet 1969)

It has been suggested that phenothiazines exert a quinidine like effect on the myocardium Antiarrhythmic activity of these drugs has been demonstrated in different types of experimental cardiac arrhythmias In an attempt to elucidate the mode of action of phenothiazines on the excitability of cardiac tissue the effect of promazine and thioridazine on the electrical behaviour of isolated rat atria was tested Both drugs (in concentrations ranging from 5×10^{6} M to 2.5×10^{5} M) exerted a negative chronotropic action they increased the threshold for excitation and for experimentally induced atr al fibrillation and they also prolonged the effective refractory period Both compounds reduced the rate of depolarization (dv/dt) of the act on potential prolonged the repolarization and in the highest concentrat ons employed they reduced the overshoot Promazine was in every respect the most potent drug The effect on the resting potential was uncertain

It is concluded that the changes in the action potential can explain the ECG disturbances in vivo and in vitro The drug induced alterations in the electrical activity of isolated cardiac tissue resembles that of quinidine local anaesthetics and other membrane stabilizing agents as defined by Shanes (1958)

REFERENCES

Landmark K Nord Med 1970 83 617 620
Landmark K A Glomste n & I Oye Acta pharmacol et toxicol 1969 27 173 182
Langslet A Acta pharmacol et toxicol 1969 27 183 192
Shanes A M Pharmacol Rev 1958 10 59 164

Asbjørn Langslet and Ivar Øye (Institute of Pharmacology, University of Oslo, Norway): SPECIFIC AND UNSPECIFIC INHIBITION OF ADRENERGIC RESPONSES IN THE RAT HEART.

In the present work we have studied the effect of d,l-propranolol, d-propranolol and chlorpromazine on the isoprenaline-induced changes in cardiac contractile force and phosphorylase activation in the isolated perfused rat heart.

As expected d,l-propranolol (10^{-7} M) completely blocked both the inotropic and metabolic response to isoprenaline (2×10^{-8} M). d-propranolol and chlorpromazine at these low concentrations had no blocking effects. However, at concentrations above 8×10^{-6} M, chlorpromazine caused a dose dependent inhibition of the isoprenaline induced (2×10^{-8} M) phosphorylase activation without concomitant inhibition of the inotropic response. Qualitatively similar results were obtained with d-propranolol. However, this agent inhibited the inotropic response to some degree.

The effect of d,l-propranolol must be expressed in terms of specific adrenoceptor blockade. The effect of chlorpromazine and partly that of d-propranolol, however, must be due to an inhibitory effect of these agents at some point distal to the β-adrenoceptor.

Probably this effect is part of the general unspecific membrane stabilizing properties of these compounds and may involve inhibition of membrane bound hormone sensitive adenyl cyclase If this holds true, the dissociation between the inotropic and metabolic responses to isoprenaline observed in this work lends support to the hypothesis that cyclic 3'5'-AMP is mediator of the metabolic response to catecholamines in the heart, but that this nucleotide is not an obligatory mediator of the inotropic response.

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Prostaglandins undergo two reactions in homogenates from lung leading to metabolites saturated at carbon atom 13 and with the hydroxyl group at carbon atom 15 oxidized to a ketone (Änggård & Samuelsson 1967) The enzymes catalyzing the reactions prostaglandin Δ13 reductase (PGR) and 15-hydroxy prostaglandin dehydrogenase (PGDH) were in the present experiments assayed in 15 different tissues of the swine

The assay of PGDH was based on the development in alkali of the 500 nm chromophore of 15 keto-PGF_1 after incubation of the tissue with PGE_1 and NAD^+ PGR was determin
PGE_1 using NADH as cofact
calculated from the initial velo
of 15 keto PGE_1 respectively

The highest activities for PGDH were found in the lung spleen and kidney Lower activities of PGDH were observed in the stomach testicle, small intestine heart and adipose tissue The renal cortex contained about three times more PGDH than the medulla The reductase was most abundant in liver spleen small intestine and kidney Fairly high activities of PGR were found in the adrenal pancreas adipose tissue brain lung and stomach The highest specific activity was however found in adipose tissue

In vivo studies have shown that in several species the prostaglandins undergo β oxidation by one or two steps as well as the reactions catalyzed by PGDH and PGR prior to excretion in urine (Samuelsson 1969) However the dehydrogenation must precede the β oxidat on since the tetranor and dinor prostaglandins are poor substrates for the PGDH (Nakano Änggård & Samuelsson 1969) The dehydrogenase reaction constitutes a biological inactivation (Änggård 1966) The wide distributions for PGDH and PGR found in the present investigation support *the concept that these enzymes catalyze* biologically important initial steps in the catabolism of the prostaglandins

REFERENCES

Nakano J E Anggård & B Samuelsson Europ J Biochem 1969 11, 386 389

Samuelsson B Proc of the 4th Int Pharmacol Meeting Basle 1969

Anggård E *Acta physiol scand 1966 66 509*

Anggård E & B Samuelsson The metabolism of prostaglandins in lung tissue Nobel Symposium 2 Prostaglandins Almqvist & Wiksell Stockholm 1967 97

Tommy Lewander (Psychiatric Research Center, Ulleråker Hospital, University of Uppsala, S-75017 Uppsala, Sweden): RELATIONSHIP BETWEEN AMPHETAMINE METABOLISM AND BRAIN CATECHOLAMINES IN THE RAT AND GUINEA PIG.

In rats a single injection of dl-amphetamine (20 mg/kg i.p.) or p-hydroxyamphetamine (20 mg/kg i.p. p-OH-A, the main metabolite of amphetamine in this species) causes a depletion of brain noradrenaline (NA) for 72-96 hours, while the parent compounds disappear from the tissue in about 24 hours.

By use of ion-exchange chromatography in combination with TLC, radioactively labelled p-hydroxynorephedrine (p-OH-NEph, the β-hydroxylated metabolite of p-OH-A) was found in the brain tissue after administration of labelled amphetamine or p-OH-A. This metabolite was present as long as brain NA was depressed by either of the two compounds.

Only small amounts of p-OH-NEph were present in the brains of reserpine treated rats given amphetamine or p-OH-A, which indicates that this metabolite is stored in NA granules as a false transmittor.

Desmethylimipramine (DMI) inhibits the p-hydroxylation of amphetamine in vivo (cf Lewander 1968a), which is followed by increased tissue levels of amphetamine (Sulser et al. 1966). In the brains of DMI pretreated rats no p-OH-NEph was found after amphetamine administration The NA levels were, however, decreased, although for a shorter duration (~12 hours).

Guinea pigs do not metabolize amphetamine to p-OH-A and accordingly p-OH-NEph was not found in the guinea pig brain. In this species the duration of the NA-depletion in brain after amphetamine was about 16 hours.

It is concluded that the prolonged depletion of brain NA in the rat is related to displacement of NA by p-OH-NEph. However, the formation of this metabolite does not seem necessary for amphetamine to cause depletion of NA. Accumulation of p-OH-NEph may contribute to the marked reduction of tissue NA levels after chronic amphetamine administration and to the development of tolerance to amphetamine (Lewander 1968b)

REFERENCES

Lewander, T : Europ. J Pharmacol 1968a, 5, 1-9
Lewander, T.: Psychopharmacologia, Berl. 1968b, 13, 394-407
Sulser, F , M. L. Owens & J V Dingell: Life Sci. 1966, 5, 2005-2010

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S OF DRUGS

It is possible to test local anaesthetic properties of drugs by an experimental arrangement based on suction electrodes The favourable recording properties of these electrodes make it possible to record high amplitude action potentials from nerve when it is fully submerged in a physiological solution

The two ends of a free dissected nerve are sucked into the openings of two fine pipette suction electrodes separated a few cm from each other The suction electrodes can be made of slender polyethylene tubes with an inner diameter roughly corresponding to the diameter of the chosen nerve Tight physical connection between the nerve trunk and the inner tube wall is important for proper functioning

Chlorinated silver wires one inside and another outside each of the polyethylene tubes form the electrical connection with the nerve The suction is *performed* and controlled by means of small syringes (1 ml) one for each electrode The two suction electrodes are used for stimulation and recording respectively *In addition they also give the nerve its necessary* mechanical support and thereby make it possible to use any standard preparation chamber at hand

Various pharmacological procedures for instance where the decrease in the amplitude of the action potential within a certain time period (30 min) is plotted against the log dose of the drug may be used (Dunlop & Shanks 1968)

The method is applied on a problem where the local anaesthetic potencies of propranolol and procaine are tested The frog sciatic nerve and the rat phrenic nerve are used in this connection

REFERENCE

Dunlop D & R G Shanks Brit J Pharmacol 1968 32 201 218

Nils Gunnar Lindquist, Sven Erik Sjöstrand and Sven Ullberg (Department of Pharmacology, Royal Veterinary College, Stockholm, and Department of Toxicology, University of Uppsala, Sweden): ACCUMULATION OF CHORIO-RETINOTOXIC DRUGS IN THE FOETAL EYE.

By means of whole body autoradiography we have found a selective accumulation and retention of certain drugs in the eyes of mice foetuses. These drugs are known to cause ocular damage in adult humans and experimental animals. This injury (toxic retinopathy) has been related to the affinity of the drugs for melanin structures of the eye (Potts 1962).

Drugs which cause ocular damage through pigment affinity belong to two categories: phenothiazines (thioridazine, chlorpromazine) and antirheumatic compounds (chloroquine).

In our investigations ^{35}S-chlorpromazine or ^{14}C-chloroquine was administered intravenously to pregnant pigmented mice. Whole body autoradiography was then performed after varying intervals following injection on either whole pregnant mice or of mouse foetuses which were surgically removed from injected mothers. The distribution pattern of injected ^{35}S-chlorpromazine was also studied in two monkeys which were autoradiographed after 4 hours and 4 days respectively.

The results showed that both ^{35}S-chlorpromazine and ^{14}C-chloroquine passed rapidly across the placental barrier in pregnant mice The drugs accumulated in the eyes of both the foetuses and the mother Pronounced radioactivity remained present in the ocular tissues for a long time (5 months) after the substances were eliminated from other tissues

A similar accumulation of ^{35}S-chlorpromazine in the melanin-containing structures of the eyes of the monkeys was observed.

In vitro experiments were also performed to investigate the drug affinity for various tissues in sections through non-injected mice Whole body sections were immersed in a water solution containing ^{14}C-chloroquine, washed, dried and autoradiographed. It was observed that ^{14}C-chloroquine showed a selective uptake in the same melanin-containing structures where intravenously injected ^{14}C-chloroquine accumulated in the living animal.

REFERENCE

Potts, A. M.: Invest. Ophthal. 1962, 1, 522-530

This work has been supported by grants from the Swedish Medical Research Council (B70-14X-2876-01A) and from Ragnar och Torsten Söderbergs Stiftelse.

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Oestrous behaviour activated by oestradiol + progesterone in the ovariectomized rat is inhibited by increased central nervous serotoninergic tone Evidence for this hypothesis has been provided by neuropharmacological studies with agents which in different ways exert an influence on monoaminerg c mechanisms (see Meyerson 1964 1968) Also muscarinic compounds like pilocarpine decrease the oestradiol + progesterone activated oestrous behaviour (Lindström & Meyerson 1967) and a relationship between central nervous monoaminergic and cholinergic inhibition of oestrous behaviour was indicated by a recent study (Lindström 1970)

In the present investigation the effect of atropine in combination with compounds with a known action on monoaminergic activity was studied on the hormone activated oestrous behaviour

Atropine (5 mg/kg s c) had no effect on oestradiol + progesterone activated oestrous behav our A clearcut inhibitory effect was however obtained *when the atropine administration was preceded by a dose of a monoamine* oxidase nhibitor (pargyline 25 mg/kg or nialamide 150 mg/kg s c) which alone had no inhibitory effect No analogous inhibition was obtained when methyl atropine (7 5 mg/kg s c) was given instead of atropine The inhibitory effect of pargyline + atropine was also present when the order of the injections of the two drugs was reversed

No significant effect was obtained by atropine (5 mg/kg s c) on the lordosis response after pretreatment with p chlorophenylalanine (400 mg/kg s c) or α methyl p-tyrosine (400 mg/kg s c) compounds considered to inhibit the biosynthesis of serotonin or catecholamines respectively (Nagatsu et al 1964 Koe & Weissman 1966)

Parallel studies on rectal temperatures revealed no obvious hyperthermic effect of atropine 5 mg/kg which could explain a possible potentiation of the pargyline effect It is therefore suggested that atropine acts more directly on mechanisms which mediate inhibition of the oestrous behaviour

REFERENCES

Koe K & A Weissman J Pharmacol exp Ther 1966 154 499 516
Lindström L Psychopharmacologia Berl 1970 17 160 168
L ndström L & B J Meyerson Psychopharmacologia Berl 1967 11 405 413
Meyerson B J Acta physiol scand 1964 63 (Suppl 241) 1 32
Meyerson B J Ann Med exp Fenn 1968 46 394
Nagatsu T M Levitt & S Udenfriend J biol Chem 1964 239 2910 2917

Nils Gunnar Lindquist, Sven Erik Sjöstrand and Sven Ullberg (Department of Pharmacology, Royal Veterinary College, Stockholm, and Department of Toxicology, University of Uppsala, Sweden): ACCUMULATION OF CHORIO-RETINOTOXIC DRUGS IN THE FOETAL EYE

By means of whole body autoradiography we have found a selective accumulation and retention of certain drugs in the eyes of mice foetuses These drugs are known to cause ocular damage in adult humans and experimental animals. This injury (toxic retinopathy) has been related to the affinity of the drugs for melanin structures of the eye (Potts 1962).

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This work has been supported by grants from the Swedish Medical Research Council (B70-14X-2876-01A) and from Ragnar och Torsten Söderbergs Stiftelse.

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ence on central nervous amines were studied To a certain exte[illegible] sexual behaviour is controlled by gonadal hormones To be able to manipulate the extent of the copulatory performance the subjects studied were castrated as adults and substituted by testosterone proprionate treatment adjusted to give a submaximal copulatory response The doses used in the present investigation ranged 0 10 - 0 50 mg/kg s c given once a week This induced willingness to mount in about 50 90 % of the animals tested Only hormone treated animals were always run parallel as controls Wistar male rats (350 400 g) were kept under reversed day night rhythm Observations were performed during the dark period under dimmed light conditions and the animals transferred to an observation cage in which the male was allowed 5 minutes of adaptation before the female was brought in As incentive animals served ovariectomized females treated with oestradiol benzoate (50 µg/animal) followed 48 hours later by progesterone (1 mg/animal) The following was measured The number of animals willing to mount and able to ejaculate time elapsed to first mount time from first intromission until ejaculation time from ejaculation to next intromission

The monoamine oxidase inhibitors pargyline (20 mg/kg s c) and nialamide (100 mg/kg s c) given 6 8 hours before the behaviour tests did not change the different sexual behaviour patterns as compared with saline treated controls The willingness to mount was however completely suppressed by pargyline (40 mg/kg s c) Pargyline (20 mg/kg i p) followed one hour later by DL 5 HTP (1 mg/kg i p) significantly reduced the number of males willing to mount compared with pargyline alone or saline treated controls The tests were conducted 1/2 hour after the DL 5 HTP injection In analogous experiments with L DOPA (1 5 or 10 mg/kg i p) no inhibitory effect was seen

A serotonin synthesis inhibitor p chlorophenylalanine reportedly induced sexual excitation in intact male rats (Tagliamonte et al 1969, Shillito 1970) The present data indicate that increased brain serotonin inhibits the willingness of the male rat to mount oestrous females

REFERENCES

Shillito E E Brit J Pharmacol 1970 38, 305-315

Tagliamonte A P Tagliamonte G L Gessa & B B Brodie Science 1969 166 1433 1435

Salvador Lluch and Gilles Fillion (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): CARDIOVASCULAR RESPONSES TO INTRAVENOUS AND INTRACORONARY ADMINISTRATION OF 5-HYDROXYTRYPTAMINE.

Some cardiovascular effects of intravenous and intracoronary injections of 5-hydroxytryptamine (5-HT) have been examined in 20 experiments on anaesthetized open-chest dogs. Atropine and Chlorisondamine, a ganglionic blocking agent, were used to eliminate the parasympathomimetic and central reflex components of the response to 5-HT. Heart rate (HR), aortic pressure (AP), left ventricular pressure (LVP), rate of rise of the left ventricular pressure (LV dp/dt) and arterial coronary blood flow (CBF) were measured before and after administration of 5-HT Intravenous injection of 5-HT (5 to 100 μ g/kg) produced a marked tachycardia accompanied by a simultaneous increase in AP, LV dp/dt and CBF. Injections of small doses of 5-HT (5 to 25 μg) into the right or the left coronary artery increased CBF moderately. LV dp/dt increased very little or remained unchanged. However, the right coronary injections induced tachycardia, while injections into the left coronary failed to elicit any change in HR. This can be explained by the direct or indirect effects of 5-HT on the pacemaker tissue of the right atrium, which is mainly supplied by the right coronary artery

These experiments show that the marked increase in AP, LV dp/dt and CBF after intravenous administration of 5-HT are due mainly to peripheral effects rather than the consequence of a cardiac component Direct injections into the coronary arteries resulted chiefly in tachycardia with little change in the other parameters The more pronounced tachycardia observed after intravenous administration as compared to intracoronary injections may be caused by the release of catecholamines by 5-HT from peripheral organs.

den)

The monoamine oxidase inhibitors MAOI, are known to potentiate the sympathomimetic effects of amphetamine (see Obianwu 1969) but opinion is divided as to the mechanism by which the MAOI exert this effect

Since amphetamine has for a long time been considered a poor substrate for MAO it was suggested that the potentiation of the amphetamine-induced effects was due to inhibition of deamination of the released noradrenaline However it was also suggested that the potentiation of the amphetamine-induced effects by the MAOI was due to inhibition of the metabolism of the drug itself rather than the released noradrenaline (see Rand & Trinker 1968)

The present studies show that the potentiation of the peripheral (contraction of the inferior eyelid and pressor response) and central (amphetamine-induced hyperactivity) effects of amphetamine in rats by the MAOI pargyline and nialamide is not accompanied by increased blood or tissue levels of the drug Pargyline and nialamide also failed to antagonize the hepatic metabolism of amphetamine in vitro whereas the tricyclic antidepressants and SKF 525 A did so

Substances which are known to antagonize the metabolism of amphetamine did not potentiate its sympathomimetic effects to the same degree as the MAOI The results provide evidence that the potentiation of the sympathomimetic effects of amphetamine by MAOI is not due to the inhibition of the metabolism of the drug

REFERENCES

Obianwu H O Acta physiol scand 1969 75 92-101

Trinker M J & F R Trinker Brit J Pharmacol 1968 33, 287 303

Bengt J. Meyerson and Asta Palis (Institute of Pharmacology, University of Uppsala, Sweden): ADVANCEMENT OF THE TIME FOR OVULATION IN THE 5-DAY CYCLIC RAT BY PILOCARPINE.

In the cyclic ovulatory rat ovulation occurs during the third night in rats with 4-day oestrous cycles and on the following night in the 5-day cycles. Rats maintained under artificial light (14 hours of light, 10 hours of darkness) as demonstrated by Everett et al. (1949) release ovulating hormone at a "critical period" which occurs 9 - 11 hours after the light is turned on the day of pro-oestrous in the 4-day animal and 24 hours later for the 5-day rat. A wide variety of drugs have been used to investigate the neural regulation involved in the anterior pituatory release of LH. Both monoaminergic and cholinergic mechanisms have been ascribed a significant role. Ovulation is blocked by anticholinergic agents and by adrenergic blocking or monoamine depleting substances provided that the compounds are given prior to the critical period (see Sawyer 1963) Experiments involving treatment with monoamine oxidase inhibitors or monoamine oxidase inhibitors in combination with DOPA or 5-HTP combined with hypophysectomy at different times during the critical period for LH discharge revealed that increased monoamine levels did not advance the period of LH in the 4-day cyclic rat. Nor was it possible to induce ovulation 24 hours early when cerebral monoamine levels were increased in the 5-day rat (Meyerson & Sawyer 1968).

In the present study pilocarpine was given on day 3 in the 5-day cyclic rat. It was shown that pilocarpine (2 x 6.25 mg/kg s.c.) administered before (12.00 and 14.00) or at a time corresponding to the critical period (14.00 and 16.00) induced ovulation 24 hours early. A single administration of pilocarpine was less effective than repeated injections The effect of pilocarpine (2 x 25 mg/kg s.c.) was prevented by atropine (2 x 5 mg/kg s.c.) given 1/2 hour before the pilocarpine injections. Pilocarpine (2 x 25 mg/kg s.c.) given after the critical period (17.00 and 19.00) was ineffective in advancing ovulation.

REFERENCES

Everett, J. W., C. H. Sawyer & J. E. Markee: Endocrinology. 1949, 44, 234-250.
Meyerson, B. J. & C H. Sawyer: Endocrinology. 1968, 83, 170-176.
Sawyer, C. H.: Advances in neuroendocrinology. Univ. Ill. Press. 1963, 444-459.

Aimo Pekkarinen (Institute of Pharmacology University of Turku Finland)

THE INHIBITING EFFECT OF THYMOLEPTIC ANTIDEPRESSANTS ON THE NEUROGENIC INCREASE OF THE CORTICOSTEROID CONTENT OF THE RAT PLASMA

The basal corticosteroid content in the quiet room was 1 hour 15 mins after *the injection of thymoleptic antidepressants* (10 mg/kg) in imipramine (10 4 ± 1 0) desipramine (13 2 ± 1 7) trimipramine (13 3 ± 1 3) dibenzepine (11 7 ± 1 3) protriptyline (13 8 ± 2 0) and Iprindol[R] groups (14 1 ± 2 0) or almost the same as in drugs in the quiet room 1 4) nortriptyline (16 0 ± basal level of the corticosteroid content of the rat plasma in the quiet room *increased very highly significantly in 15 mins in the dog room* to 46 9 ± 0 8 µg % (109 rats) and in 60 mins to 43 3 ± 0 7 µg % (143 rats) (I) This neurogenic increase of the corticosteroid content was often very highly significantly inhibited by thymoleptics (10 20 mg/kg) With 20 mg/kg dose the corticosteroid content was smallest in the dog room in the amitriptyline group (16 1 ± 2 5 µg %) as well as in the trimipramine desipramine and Iprindol[R] groups 17 7 ± 1 4 19 2 ± 2 1 and 20 3 ± 1 9 µg % resp in 60 mins and often less than one half from that in the dog room without these drugs With this dose the corticosteroid content in the dog room was in the nortriptyline and dibenzepine groups 24 1 ± 3 3 and 23 7 ± 1 5 µg % resp (II) With a smaller dose (10 mg/kg) of thymoleptics the corticosteroid content was then very highly significantly reduced in all groups (protriptyline to 15 9 ± 1 1 µg % Iprindol[R] 17 2 ± 1 8 µg % trimipramine 20 2 ± 1 7 µg % imipramine 21 0 ± 1 9 µg % amitriptyline 21 0 ± 2 1 µg % desipramine 25 7 ± 1 4 µg % nortriptyline 28 4 ± 2 4 µg % and doxepine groups 33 9 ± 2 2 µg %) (III) The small doses of thymoleptic drugs (5 and 2 5 mg/kg) also usually reduced significantly the neurogenic increase of corticosteroid content in 60 mins although less than the higher doses (IV) The corticosteroid content was very highly significantly reduced by all 9 thymoleptics (10 mg/kg) already in 15 mins in the dog room in the protriptyline (26 3 ± 1 4 µg %) amitriptyline (26 6 ± 2 3 µg %) trimipramine (31 1 ± 1 5 µg %) desipramine (25 8 ± 1 6 µg %) nortriptyline (27 2 ± 1 3 µg %) dibenzepine (30 4 ± 1 7 µg %) and doxepine groups (32 8 ± 2 0 µg %) This inhibition of *neurogenic increase of corticosteroid content* seems to be somewhat specific for thymoleptics

J. B. Osnes, J. Mørland, T. Christoffersen and I. Øye (Institute of Pharmacology, University of Oslo, Norway): SPECIFIC DETERMINATION OF LABELLED CYCLIC 3'5'-AMP.

Specific determination of labelled cAMP is a prerequisite for measuring the activity of the enzyme adenyl cyclase when radioactive ATP is used as substrate. Usually a number of labelled metabolites are formed, some of which are difficult to separate from cAMP by simple chromatographic procedures The number and the relative amounts of these metabolites depend on the tissue and the incubation conditions used.

The source of adenyl cyclase in the present experiments was a coarse particle fraction from rat liver homogenate. The incubation mixture (0.4 ml) contained 25 mM Tris buffer, pH 8.3, 10 mM theophyllin, 6 mM $MgCl_2$, 4 mM ATP, ^{14}C-ATP, 0.3 mM cAMP, ^3H-cAMP (to calculate recovery), enzyme preparation (10.7 mg prot./ml) and in some cases either 10mM NaF, $1.4 \cdot 10^{-2}$ mM glucagon or $9.5 \cdot 10^{-2}$ mM epinephrine. Incubation was carried out at 30 °C for 10 min and terminated by heating. The supernatant from the incubation mixture was chromatographed on Whatman paper no. 1 for 16 - 20 hours at 19 °C with a solvent system consisting of 96 % ethanol - 2 M ammonia - 2 M acetic acid (10:1.9:2.1), pH 7.15. The cAMP was completely separated from ATP, ADP, and 5'-AMP and incompletely separated from some other labelled metabolites Most of the ^{14}C-radioactivity in the cAMP zone of the chromatogram originated from adenosine-tailing The eluate from this zone (1 ml of 10 mM glycylglycin buffer, pH 7.4, 1 mM $MgCl_2$) was incubated with 100 µg heart cyclic-phosphodiesterase (Boehringer) for 30 min at 37 °C. The incubation mixture was rechromatographed in the system described above. The labelled 5'-AMP (originating solely from cAMP) now migrated to a zone devoid of labelled contaminants. ^{14}C- and ^3H-radioactivities of this zone were counted in a liquid scintillation spectrometer and the amount of cAMP calculated

Counting of the different zones of the remaining part of the second chromatogram revealed various amounts of ^{14}C-labelled metabolites completely separated from the 5'-AMP. The amounts of contaminants differed with the adenyl cyclase activator used and might thus cause systematic errors in the evaluation of the adenyl cyclase activity if not completely removed

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HEORETICAL CON
TRATION AS DETER-
MINED BY THE ABSORPTION AND ELIMINATION RATE CONSTANTS

The blood concentration curve of a drug administered orally may with a few exceptions be represented by a two compartment model



from which follows the equation

$$C = A \frac{k_1}{k_2 \; k_1} (e^{\;k_1 t} \; e^{\;k_2 t})$$

The maximum concentration C_{max} is by derivation found to be

$$C_{max} = A \; F \quad \text{where } F = (\frac{k_1}{k_2})^{\frac{k_2}{k_2 \; k_1}}$$

and A = the concentration obtained by i v injection of the same dose

If F is plotted against k_1/k_2 it is found that a reduction of the absorption rate constant which changes the ratio from 1000 1 to 100 1 reduces C_{max} by 3 6 % only whereas a reduction from 10 1 to 1 1 reduces C_{max} by 53 %. This might to some extent explain why a large difference in dissolution rate constants found in vitro is not always reflected in a similar difference in the in vivo behaviour of the drug The time of the occurrence of C_{max} is likewise altered by alterations of k_1/k_2 but in this case the absolute values are of mportance too Aga n comparat vely large changes in k_1 as determined in vitro may give only minor changes in the resulting concentration curve

A. D. Popóva*, S. A. Slorach and B. Uvnäs (Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden): THE RELATIONSHIP BETWEEN THE UPTAKE OF TOLUIDINE BLUE AND CHLORPROMAZINE AND THE RELEASE OF HISTAMINE FROM ISOLATED RAT MAST CELL GRANULES.

Rat mast cell granules stain metachromatically with toluidine blue (TB) a reaction which has long been used for the identification of mast cells. Smith (1958) showed that the dye releases histamine from mast cells in situ. Popóva (1965) demonstrated that when treated with chlorpromazine (CPZ) mast cells stained supravitally with TB lost their colour and the mast cell granules acquired a strong fluorescence, due to accumulation of the drug.

The present study is an investigation of the binding of TB and CPZ to isolated mast cell granules and of the displacement of histamine from histamine-loaded granules by these two substances.

Isolated histamine-loaded rat mast cell granules released their histamine on exposure to TB: maximum histamine release and TB uptake occurred at the same TB concentration ($5 \cdot 10^{-4}$ M). Similarly, $5 \cdot 10^{-4}$ M CPZ gave maximum release of the granule histamine from histamine-loaded granules. The maximal uptake capacities for TB and CPZ were considerably greater than that for histamine. In addition, TB and CPZ were able to bind to granules at lower pHs than histamine.

On exposure to increasing concentrations of CPZ, isolated TB-loaded granules progressively released the dye and took up CPZ. There was a quantitative correlation between TB release and CPZ uptake. Maximum TB release took place at a CPZ concentration of $5 \cdot 10^{-3}$ M, i.e. ten times that required for maximum histamine release. These results indicate that the loss of colour from TB-loaded cells exposed to CPZ is due to a quantitative exchange of TB from its binding sites on the granules for CPZ

Furthermore, these observations suggest that TB and CPZ are able to bind to other cationic sites on the granules in addition to those which bind histamine.

REFERENCES

Popóva, A D : Compte Rendus Acad Bulg Sci 1965 18, 1063-1066
Smith, D. E Amer J. Physiol 1958 193, 573-575

* Visiting scientist from Institute of Physiology, Department of Pharmacology, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Research Laboratories AB

MACOLOGY OF LEO 640 A
SSANT

In the search for a new antidepressant the pharmacology of Leo 640 (N-methyl N (4 chlorobenzoyl methyl) 3 (10 11 dihydro 5H dibenz(b f)azepin-5 yl) propylamine hydrochloride) has been studied Acute toxicity (48 h) In m ce LD50 p o > 2500 mg/kg LD50 i p 920 mg/kg, LD50 s c > 1000 mg/kg In rats LD50 p o > 1000 mg/kg LD50 i p > 1000 mg/kg LD50 s c > 1000 mg/kg The ED50 values in antireserpine tests in mice

tion in mice was 8 mg/kg p o Blood pressure studies in urethan chloral ose anaesthetized cats revealed an antagonism to the pressure effects of tyramine with < 2 mg/kg intraduodenally MAO inhibition could be exclud ed by studying 5 hydroxytryptophane potentiation in rats and serotonin con tent in rat brains No anticholinergic properties could be shown in mice in tests studying oxotremorine tremor apomorphine potentiation mydriasis and with acetylcholine induced bronchospasm in guinea pigs The ED50 for the antagonism to oxotremorine induced hypothermia in mice was 2 mg/kg p o In rats the majority of symptoms induced by an i v injection of pilo carp ne (salivation lacrimation piloerection tremor chewing and hunch ing of the back) were not affected but scratching with the hindlegs induced by the same substance could be inhibited No effects could be shown in mice in studies of the motility or hexobarbitone and alcohol potentiation Hist am ne bronchospasm in guinea pigs could not be influenced These results were cons dered so interesting that preclinical studies in humans were started which gave promising results (Siwers et al 1969)

REFFRENCES

Siwers B D Tuck U Freyschuss D L Azarnoff & F Sjöqvist Proc Fourth International Congress on Pharmacology 1969 p 432

Leif Rentzhog (Institute of Pharmacology, University of Uppsala, Sweden):
DOUBLE ISOTOPE DERIVATIVE ASSAY OF CATECHOLAMINES.

A double isotope derivative dilution technique for quantitative determination of catecholamines has been developed. The quantification of endogenous amines is based on the isotope ratio of purified triacetylated derivatives.

Tracers of ^{14}C labelled catecholamines are added to plasma or tissue. The tracer and endogenous amines are isolated on alumina using a batch procedure. This eliminates substances that otherwise may interfere with the acetylation process and give a high background. The eluates are lyophilized, redissolved in 0.2 ml of 0.5 N acetic acid and acetylated with 10 micromoles of acetic anhydride ^{3}H in the presence of a slight excess (10 - 15 mg) of sodium bicarbonate. The acetylation procedure takes a few minutes and after destruction of remaining sodium bicarbonate with dilute acetic acid unlabelled catecholamine triacetate carriers are added. The acetylated amines are extracted with chloroform, after which they are isolated and purified by repeated thin-layer chromatography.

Constancy of $^{3}H:^{14}C$ isotope ratio on successive chromatograms is taken as a proof of the derivatives having been separated from impurities. In some experiments the chromatographic procedure has been followed by crystallization to confirm the stability of the isotope ratios.

The advantage of the method is that small amounts of one catecholamine can be determined in the presence of a large excess of others.

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in the cage The
this night perch

The circadian period was counted from the time the bird left the night perch (waking up) and usually consisted of one period of l ght (activity) and one period of darkness (rest) (Wahlström 1964)

Single doses of ethanol (0 5 3 0 g/kg in a constant volume of 10 ml/kg) were given by gavage either early (AM) or late (PM) in the self selected period of activity All changes after these doses were calculated as a dif ference from an average obtained from the five circadian periods immediately prior to the experiments

In the PM series no certain effect was recorded in the circadian period in which ethanol was administered There was however a consistent de crease in the duration of activity in the next circadian period Since the duration of this circadian period was uninfluenced the decrease in activity was due to an earlier roosting In the different series the following decreases in hours were recorded

Dose	Decrease	S E M	n	Dose	Decrease	S E M	n
0 5	0 79	0 46	12	1 0	0 91	0 42	9
1 5	0 62	0 35	14	2 0	1 45	0 67	7
2 5	0 74	0 40	9	3 0	0 75	0 27	8

In the AM series a corresponding change (1 27 $\pm$ 0 44 h n = 11) was seen only with a dose of 3 0 g/kg No consistent changes were seen with lower doses n the AM series

No immed ate effect of ethanol at least with doses lower than 3 g/kg could be established The cons stent change seen the day after ethanol is by de f n tion a hangover effect Whether this phenomenon is related to the well known human experience s uncertain In both cases however there seems to be a common tendency to go to bed early

REFERENCE

Wahlström G Acta Soc med Uppsala 1964 69 241 271

Th s work has been supported by the Tri Centennial Fund of the Bank of Sweden

Oskar Rohte (Department of Pharmacology, Research Laboratories, AB Leo, Hälsingborg, Sweden): THE EFFECTS OF PSYCHOTROPIC DRUGS ON THE GROOMING BEHAVIOUR OF RESERPINIZED WHITE MICE.

The ptosis and the grooming movements of reserpinized white mice (0.6 mg reserpine per kg i.v.), dusted with pulverized charcoal, were studied under the influence of the following psychotropic drugs. d-amphetamine, desipramine, Leo 640, imipramine, amitriptyline, nortriptyline, trimipramine, iproniazid. The test substances were given twice perorally, the first dose at the same time as reserpine, the second 16 hours later. According to a test described earlier (Rohte 1969), the intensity of grooming was determined by grading the blackening with a three-point scale. Ptosis was graded with a key described by Rubin (1957). Only d-amphetamine, desipramine and Leo 640 (a new potential antidepressant) counteracted the reserpine-induced decrease of grooming movements. Ptosis was antagonized by all substances with the exception of nortriptyline and trimipramine In order to find the position of this new test in the test-battery the results were compared with those obtained in the reserpine-temperature test in mice (Askew 1963), where the test substances were given only once about 16 hours after reserpine, and the ptosis test in mice (Domenjoz & Theobald 1959), where the test substances were given one hour before reserpine With the exception of imipramine there was a parallelism between the results of the reserpine grooming test and the temperature test. The results of the ptosis test, where the substances were given prophylactically, showed positive effects with all the substances investigated with the exception of trimipramine. This indicates that this test is the most sensitive one, even if it is known that it only measures peripheral antireserpine effects (Fielden & Green 1965).

For a further classification of the effects of antidepressants on the CNS, the grooming test might be a useful tool

REFERENCES

Askew, B M : Life Sci 1963, 2, 725-730
Domenjoz, R & W Theobald Arch int Pharmacodyn 1959 120, 450-489
Fielden, R & A L Green: J Pharm Pharmacol 1965 17 185-186
Rohte, O., Psychopharmacologia, Berl 1969, 14 18-22
Rubin, B M H Malone, M H Vaugh & J C Burke J Pharmacol 1957, 120, 125-136

The canaries are kept singly in light proof cages The l ght inside is extin guished as long as the bird sits on one of the two perches in the cage The bird can thus choose darkness in the cage by hopping onto this night perch The circadian period was counted from the time the bird left the night perch (waking up) and usually consisted of one period of l ght (activity) and one period of darkness (rest) (Wahlström 1964)

Single doses of ethanol (0 5 - 3 0 g/kg in a constant volume of 10 ml/kg) were given by gavage either early (AM) or late (PM) in the self selected period of activity All changes after these doses were calculated as a dif ference from an average obtained from the five circadian periods immediately prior to the experiments

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Th s work has been supported by the Tri Centennial Fund of the Bank of Sweden

Åke Ryrfeldt and Eskil Hansson (Toxicology Laboratories, AB Astra, Södertälje, Sweden): THE BILIARY EXCRETION OF SOME QUARTERNARY AMMONIUM COMPOUNDS AND TERTIARY AMINES IN RAT.

Labelled quarternary ammonium compounds and tertiary amines were injected into the femoral vein on bile fistula rats (Sprague-Dawley) and bile was collected at hourly intervals for 4 - 6 hours. The animals were under pentobarbital anaesthesia (30 mg/kg i.p.) during the experiment. The radioactivity of the bile samples were assayed by liquid scintillation technique and TLC or paper chromatography were performed on aliquots of bile samples. The following quarternary ammonium compounds were investigated: 3-acetylpyridine methiodide-^{14}C (Mw 136), 1-(3-pyridyl) ethanol methiodide-^{14}C (Mw 138), nicotine isomethionineiodide-^{14}C (Mw 177), cotinine methiodide-^{14}C (Mw 206), emepronbromide-^{14}C (Mw 283), succinylcholine-^{14}C (Mw 291), methylatropine-^{3}H (Mw 303), N-hydroxyethylpromethazine-^{35}S (Mw 330), and piribenzil-methylsulphate-^{3}H (Mw 355). The tertiary amines nicotine-^{14}C (Mw 162), lidocaine-^{14}C (Mw 234), mepivacaine-^{14}C (Mw 246), amitriptyline-^{14}C (Mw 277), imipramine-^{14}C (Mw 284), and prometazine-^{35}S (Mw 284) were used in the investigation. The molecular weights are calculated on the free onium compound and amine

Only small amounts (0.0 - 2.7 %) of the injected doses of activity were eliminated in the bile with compounds of molecular weight up to about 200, both with quarternary ammonium compounds and tertiary amines Compounds above a molecular weight of 200 showed high excretion in the bile (18 - 54 %). Chromatographic studies indicated that most of the excreted amounts in the bile were attributed to metabolites. Succinylcholine showed to be an exception since only 0 4 % of the injected dose was excreted in the bile. The results indicated that a correlation may exist between the amount of excretion into the bile and the molecular weight of the injected compound The compounds seem to undergo metabolic transformation(s) before elimination into the bile.

There is evidence to indicate that tricyclic antidepressants block preferentially the uptake of either NA or 5HT into central monoaminergic neurons ... that some tricyclic antidepressants

to enhance the NA pressor response
of analgesia and the inhibition of NA uptake ...
used for the measurement of pain and analgesia The pressor responses to injected NA were recorded in conscious rabbits chronically catheterized into the femoral artery and vein

The main results are summarized as follows

Drug (mg/kg iv)		Analgesic activity	Increase of morphine analgesia	Increase of NA pressor response
Imipramine	5 0	+	++	++
Desipramine	2 5	0	0	+++
	5 0	+	+	+++
Trimipramine	4 0	++	++	0
Amitriptyline	2 0	++	+++	0
Nortriptyline	2 5	+	0	+
Protriptyline	3 0	+	+	+++
Dibenzepine	3 0	0	+	++
Opipramol	5 0	0	0	0
Doxepine	2 5	++	+++	0
Iprindole	8 0	0	Decrease	0

The results demonstrate a dissociation between analgesia and the NA response potentiation This suggests the ability of blocking the NA uptake may be unrelated to analgesic effect Considering our animal data against clinical exper ences with tricyclic antidepressants on various parameters of depressive syndromes tha analgesic activity fairly correlates with their anxiolytic effect and the NA potentiation with their psychomotor stimulant action in depressive patients

REFERENCES

Carlsson A K Fuxe & U Ungerstedt J Pharm Pharmacol 1968 20 150 151
Mattila M J & L Saarnivaara Ann Med exp Fenn 1968 46 72 77
Saarnivaara L Ann Med exp Fenn 1969 47 180-190

Grant S gr d Juselius Stiftelse

J. Schuberth, B. Sparf and A. Sundwall (The Psychiatric Research Center, Ulleråker Hospital, Uppsala, and the Department of Pharmacology and Toxicology, Research Department of the KABI Group, Stockholm, Sweden):
DISTRIBUTION AND METABOLISM OF RADIOACTIVE CHOLINE IN THE MOUSE WITH SPECIAL REFERENCE TO THE BIOSYNTHESIS OF ACETYLCHOLINE.

It has recently been shown that radioactive acetylcholine (ACh) is rapidly formed in the brain (Schuberth et al. 1969) when ^{3}H-choline (^{3}H-Ch, 0.5 µ moles, 100 µ Cie) is given intravenously to mice. The present report gives further information on the fate of intravenously injected choline.

From about 10 to 60 min. the radioactivity in plasma is rather constant. The concentration in salivary gland, liver and heart is 10 - 20 times higher than in plasma. In brain, however, the concentration of radioactivity during the time interval 2 - 240 min. is about the same as the plateau concentration established in plasma.

The rate of metabolism was highest in the liver and salivary gland (only 4 - 5 % of the radioactivity was in the form of unchanged Ch 5 min. after injection). The metabolism was much slower in brain and heart (37 and 62 % unchanged after 5 min.) The time course of unchanged choline in plasma is quite different from that of total ^{3}H. Following a rapid mixing phase the concentration decreases exponentially with a half-life of 7 min. (from 3 to 25 min.) whereafter the rate is much slower. The concentrations of ^{3}H-Ch in plasma and brain are very similar during the time interval 2 - 40 min The other radioactive acid extrable compounds revealed by high voltage electrophoresis and paper chromatography have been identified as phosphorylcholine (PhCh) betaine and acetylcholine (ACh). Radioactive PhCh was rapidly formed in all tissues examined (except plasma). A radioactive compound which presumably is identical with betaine was found only in liver and salivary gland.

Rather large amounts of radioactivity were found in the presumed ACh spot following reinecke precipitation and electrophoresis of acid extracts from brain, stomach, salivary gland and heart. The identity of ACh was checked by paper chromatography and by performing electrophoresis of tissue extracts hydrolysed with choline esterase (ChE)

REFERENCE

Schuberth, J., B Sparf & A Sundwall, J Neurochem 1969, 16 695-700

No 63

J Schuberth B Sparf and A Sundwall (The Psychiatric Research Center, Ulleråker Hospital Uppsala, and the Department of Pharmacology and Toxicology Research Department of the KABI Group, Stockholm, Sweden)

THE EFFECT OF DRUGS ON THE TURNOVER OF ACETYLCHOLINE

It is well known that the level of acetylcholine (ACh) in the brain is affected by a variety of drugs By studying the turnover of ACh significant differences between the drugs can probably be revealed and also enable differentiations between effects on biosynthesis, storage or release of the nerve transmittor

The turnover has been estimated by measuring the formation of labelled ACh in mouse brain from intravenously injected labelled choline (Ch) after administration of different drugs That brain may be dependent on circulating Ch for the synthesis of ACh has earlier been indicated by the rapid synthesis of radioactive ACh in the nerve endings following intravenous injection of radioactive Ch (Schuberth et al 1970)

The conversion of Ch to ACh was found to be markedly inhibited by Nembutal anaesthesia and by oxotremorine Both these drugs produce a reduction of the body temperature in mice However the reduced rate of synthesis of

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the effect of anaesthesia on ACh synthesis is rather specific

As the level of endogenous ACh in the brain is increased during anaesthesia and oxotremorine treatment the conclusion must be that anaesthesia and oxotremorine is accompanied by a reduced utilization of ACh

REFERENCE

Schuberth J B Sparf & A Sundwall J Neurochem 1970 17 461-468

Guldborg Serck-Hanssen (Institute of Pharmacology, University of Oslo, Norway): IMPAIRED IN VITRO LABELLING OF PROTEIN IN ADRENAL MEDULLA SLICES ON INHIBITION OF THE CATECHOLAMINE SYNTHESIS.

Secretion of catecholamines, CA, from the adrenal medulla leads to accelerated synthesis of CA (Gordon et al. 1966) This is explained by increased activity of tyrosine hydroxylase released from end product inhibition caused by CA (Spector et al. 1967) as well as elevated levels of the enzyme (Mueller et al. 1969) Protein, believed to be part of the complex binding CA in the chromaffin granules, is secreted together with the CA (Banks & Helle 1965). Resynthesis of granular protein is therefore to be expected as well In rats exposed to cold stress incorporation of labelled amino acids into medullary protein is stimulated (unpublished results). This suggests a correlation between synthesis of CA and protein, and it was of interest to see whether protein synthesis in this tissue is dependent on concomitant CA synthesis.

Incorporation of ^{3}H-lysin into protein in slices of bovine adrenal medulla, incubated in Krebs Ringer bicarbonate buffer in the presence and absence of α-methyl-p-tyrosine, α-MPT, has been measured. This compound is known to inhibit tyrosine hydroxylase; the rate limiting enzyme in CA synthesis. The presence of α-MPT in concentrations varying between 0.5 - 1.25 mM lowered the incorporation into protein about 20 - 25 % compared with the control The presence of the inhibitor in a concentration of 1.25 mM caused no decrease in the incorporation of lysin into protein in slices of cortex from the same glands. This indicates that the observed effect in the medulla is not due to an interference by the amino acid analogue with the protein synthesizing machinery

REFERENCES

Banks, P. & K Helle: Biochem J 1965, 97, 40C-41C

Gordon, R , S Spector, A Sjoerdsma & S Udenfriend: J Pharmacol exp. Ther. 1966, 153, 440-447

Mueller, R A , H Thoenen & J Axelrod: Molec Pharmacol 1969, 5, 463-469.

Spector, S , R. Gordon, A. Sjoerdsma & S Udenfriend: Molec Pharmacol. 1967, 3, 549-555.

Annette Skinhoj (Department of Pharmacology Royal Danish School of Pharmacy Copenhagen Denmark) THE BLOOD PRESSURE EFFECT OF ANGIOTENSIN IN NEPHRECTOMIZED RATS

Ang otensin injected intravenously into normal rats in a total amount of 2 5 7 5 µg/kg (Hypertensin Ciba) causes a rise in the blood pressure throughout the injection period, i e 7 15 min and less than 5 min after cessation of the injections the blood pressure returns to the initial level When administering the same supramaximal dose of angiotensin to rats nephrectomized 24 hours before and anaesthetized with urethane the increase of the blood pressure will last for more than 20 min after discontinuation of the injections The prolonged effect is not due to the lack of a renal elimination of angiotensin because it is not seen in recently nephrectomized rats

Injection of a subpressor dose of angiotensin during 1 hour prevents the long lasting blood pressure effect of the subsequent administration of a supramaximal dose This suggests that the altered response is provoked by the lack of endogenous angiotensin due to the nephrectomy and not by the 'unspecific uremic state

The prolonged rise in blood pressure is not seen in animals pretreated with adrenergic α blocking agents which indicates an interference with the catecholamine stores Removal of the adrenals with the kidneys or destruction of the spinal cord by pithing the animals also prevent the prolongation whereas pretreatment with ganglionic blocking agents has no effect

Guldborg Serck-Hanssen (Institute of Pharmacology, University of Oslo, Norway): IMPAIRED IN VITRO LABELLING OF PROTEIN IN ADRENAL MEDULLA SLICES ON INHIBITION OF THE CATECHOLAMINE SYNTHESIS.

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REFERENCES

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Angiotensin injected intravenously into normal rats in a total amount of 2 5 - 7 5 μg/kg (Hypertensin Ciba) causes a rise in the blood pressure throughout the injection period, i e , 7 - 15 min. and less than 5 min. after cessation of the injections the blood pressure returns to the initial level. When administering the same supramaximal dose of angiotensin to rats nephrectomized 24 hours before and anaesthetized with urethane the increase of the blood pressure will last for more than 20 min after discontinuation of the injections The prolonged effect is not due to the lack of a renal elimination of angiotensin because it is not seen in recently nephrectomized rats

Injection of a subpressor dose of angiotensin during 1 hour prevents the long lasting blood pressure effect of the subsequent administration of a supramaximal dose This suggests that the altered response is provoked by the lack of endogenous angiotensin due to the nephrectomy and not by the "unspecific uremic state"

The prolonged rise in blood pressure is not seen in animals pretreated with adrenergic α-blocking agents which indicates an interference with the catecholamine stores Removal of the adrenals with the kidneys or destruction of the spinal cord by pithing the animals also prevent the prolongation, whereas pretreatment with ganglionic blocking agents has no effect.

R. B. Sund (Department of Pharmacology, Institute of Pharmacy, University of Oslo, Norway): ESTIMATION OF ATROPINE AND SOME ANTICHOLINERGICS CHEMICALLY RELATED TO ATROPINE, ON THE ISOLATED RAT JEJUNUM.

Atropine and the below mentioned anticholinergics have been bioassayed on the surviving rat jejunum bathed in aerated Tyrode solution of 35 °, containing methysergide maleate 10 - 25 ng/ml. 4 cam long intestinal samples were taken 5 - 10 cm distal to the duodeno-jejunal flexure and throughout the entire jejunum. The sample, washed free of intestinal content, was stimulated every 3rd minute by a submaximal dose of acetylcholine. Contractions were recorded with a classical isotonic lever on a smoked drum. Previous to every 2nd stimulation, atropine was allowed to act on the tissue for 60 seconds (adding atropine more frequently than this lead to hangover effects) The reduction in amplitude of the following contraction was measured and used for calculation. Doses of atropine sulfate of 1.5 - 4 ng/ml always caused a significant degree of inhibition. Some further increase in sensitivity could be obtained by prolonging the atropine contact period.

10 "unknown" solutions of atropine sulfate (being 0 75 - 1.33 x the strength of the standard solution) were assayed in randomized dose pair design, with a total of 8 dose pairs per assay. Mean deviation from the theoretical content was -0.5 % (range -6.8 % - +8.3 %). The activity of l-hyoscyamine sulfate compared to atropine sulfate was 1.9. The relative potencies (atropine sulfate = 1) of the following atropine analogues were, when tested in (3 + 3)-assays with 4 - 6 groups of doses: Methylscopolamine · HNO_3 1.96, methylatropine · HNO_3 1.13, scopolamine · HBr 0.67 methylhomatropine · HBr 0.029, homatropine · HBr 0.016, butylscopolamine · HBr 0.0037. The latter compound has been claimed to act mainly by ganglionic inhibition and not by peripheral myoneural blocking.

The described method has later on been used to evaluate the intestinal absorption of atropine at various concentrations and pH levels

A Sundwall K Lthne and J Vessman (Department of Pharmacology and Toxicology and Department of Analytical Chemistry Research Department of the KABI Group Stockholm Sweden) METABOLIC DISPOSITION OF EMEPRONIUM IN ANIMALS AND MAN WITH A NOTE ON THE RELATIONSHIP BETWEEN PLASMA CONCENTRATION AND EFFECT

Emepronium bromide (Cetiprin[R] ethyl (3 3 diphenyl 1 methyl propyl) dimethyl ammonium bromide) is a quarternary ammonium compound with anticholinergic and ganglion blocking properties The drug is rather extensively metabolized in the dog following both intravenous, oral and rectal administration After oral administration most of the radioactivity in the urine is in the form of metabolites Following intravenous injection twice as much label was found in faeces as in urine about half in the form of the unchanged drug indicating a rather extensive enterohepatic circulation

0 4 1 6 % of the dose were excreted unchanged in the urine following oral administration The results from the experiments with the radioactive compound indicated however that about 6 % of an oral dose is absorbed when metabolism and biliary excretion is accounted for In man about 1 % of an oral dose is recovered unchanged in the urine within 24 hours

Although the lethal doses differ between rat and dog (1100 and 250 mg/kg) lethal plasma concentrations were found to be almost the same (17 and 24 μg/ml) In the dog threshold concentration for effect on heart rate is of the order 50 100 ng/ml Clinically effective doses (150 mg orally) produce peak plasma concentrations of 15 to 100 ng/ml (Vessman et al 1970) In the studies on the absorption of emepronium in man it was noticed that side react ons such as cycloplegia occurred at plasma levels of 1 0 μg/ml

Relationship between plasma concentrations of emepronium and effect in different species

Species	LD_{50} mg/kg	Lethal plasma concentration μg/ml	Effective dose mg/kg	Effective plasma concentration ng/ml
Rat	1100	17		
Dog	250	24	20	50 - 100
Man			1 2	15 - 100

REFERENCE

Vessman J S Strömberg & G Rietz Acta pharm Suecica 1970 Submitted for publication

Anja H. Tissari and E. Marjatta Raunu (Department of Pharmacology, University of Helsinki, Finland): FORMATION AND ELIMINATION OF 5HIAA IN DEVELOPING BRAIN.

A newborn rat has only 1/3 of adult brain 5HT but an almost adult 5HIAA level. In the rabbit brain both contents increase simultaneously.

MAO block was caused in 1-day-old and adult rats by administering either 5 mg/kg tranylcypromine once or 100 mg/kg pargyline at 0 and 50 mg/kg at 3 hours i.p. (100 mg/kg twice to adults). At 2 hours the proportional brain 5HT contents were similar in both drug and age groups, i.e. more than twice the control level. In 1-day-olds then, tranylcypromine caused a higher 5HT increase for over 9 hours, approaching 7 times the control level. In the adults the increase of brain 5HT levelled off after 6 hours, being 3 times the control level after tranylcypromine and 4 times after pargyline treatment. 2 hours after treatment the brain 5HIAA content of the 1-day-old rats had declined to 39 % of control with tranylcypromine, and to 42 % with pargyline and that of the adults to 34 % and 28 %, respectively. The minimum brain 5HIAA level attained by pargyline was 26 % in the 1-day-olds and 14 % in the adults. From 3.5 hours on motor activity was strongly increased in the 1-day-old rats after both and in the adult rats after pargyline treatment.

200 mg/kg probenecid was administered i.p. twice, at 0 and 1 hour. The brain 5HIAA content of 1-day-old rats was 108 % of control at 1.5 hour and 152 % at 3 hours, the adult contents being 180 % and 260 %. The brain 5HT and blood 5HIAA and 5HT contents did not change. Thus like 5HIAA transport, probenecid access to the brain may be deficient in young rat. Strong sedation was observed in the adult rats, perhaps due to accumulation of some 5HT metabolite, e.g. 5-hydroxyindoleacetaldehyde. No behavioural changes and a low brain 5HIAA level were observed after similar probenecid treatment to adult guinea pigs.

Passive diffusion may contribute to the decline of brain 5HIAA after MAO block in the 1-day-old rats, since 5HIAA administration (100 mg/kg i.p.) increased it 25 times, compared to 5 times in the adult rats. There may also be some insufficiency in the 5HT binding capacity of 1-day-old rat brain. When its 5HT content was elevated 4 times by tranylcypromine and 8 times by 5HTP administration, the proportional 100 000 g supernatant 5HT content rose from 18 % to 30 % and 51 % respectively. In the adult brain the increase was from 25 % to 29 % and 35 %.

Brain 5HIAA accumulation after probenecid reached adult level 3 weeks and subcellular distribution of elevated brain 5HT 5 weeks after birth. The ratio of brain 5HIAA to 5HT content also reached the adult value then.

n T k Be t l Hamberger and Folke Sjöqvist (Department of Psychiatry

Plasma from patients treated with nortriptyline (25 50 mg t i d) markedly inhib ts the uptake of ^{3}H NA in adrenerg c nerves of the isolated iris of the rat incubated in plasma with addition of ^{3}H NA 10^{-7} M (Borgå et al 1970) The same procedure was used to study the effect of various thymoleptic and

potential antidepressant Leo 640 in which one N methyl group is substituted for a p chlorobensoyl

However plasma from patients treated with opipramol (50 100 mg t i d) a tricyclic compound with purported antidepressant effect did not inhibit ^{3}H NA uptake nor did plasma drawn from patients treated with thiorida zine chlorprotixene trifluoperazine and haloperidol Chlorpromazine plasma produced either no effect or (in certain individuals on high doses) a moderate inhibition of the uptake of ^{3}H NA These results will be discussed in relation to the clinical pharmacology of thymoleptic and neuroleptic drugs

REFERENCE

Borgå O B Hamberger T Malmfors & F Sjöqvist Clin Pharmacol Ther 1970 In Press

This study was supported by grants from the Swedish Medical Research Council and the National Institutes of Health Bethesda U S A (GM 13978 05)

Institute of Pharmacy,

. . . ᵖFECTS OF DISULFI- . . . . .L ON FACTORS OF THE PLASMA KININ SYSTEM.

Disulfiram (tetraethylthiuramdisulphide) and diethyldithiocarbamate were tested for kininase inhibiting effect (in vitro-experiments). According to Eldjarn(1950) and Strømme (1964) disulfiram in the organism is readily reduced to diethyldithiocarbamate. Plasma kininase and erythrocyte kininase preparations from human blood were used, and the inactivation of bradykinin in Tris-buffered incubation mixture was determined under standard conditions. Disulfiram was found to be a potent inhibitor for human erythrocyte kininase (inhibition obtained at 25 μ g/ml), but did not inhibit human plasma kininase (in concentrations up to 200 μ g/ml). Diethyldithiocarbamate was a potent inhibitor for human plasma kininase (inhibition obtained at 25 - 50 μ g/ml), but had a weaker effect on erythrocyte kininase (100 - 200 μ g/ml being required for inhibition)

Ethanol in the concentration range 5 - 10 % (w/v) was found to release considerable amounts of kinin in human citrated plasma stabilized with EDTA-2Na, 4 mg/ml Ethanol 10 % (w/v) was found to release the total amount of kinin in human plasma substrate in 3 - 5 hours. In minor concentrations (0.5 - 4 % (w/v) were tested) ethanol significantly potentiated the kinin releasing effect of ellagic acid (in concentrations of 2 and 4 10^{-5} M) Ethanol in the same concentration range also showed a tendency to potentiate the kinin releasing effect of plasma kallikrein, but the effect was not significant.

REFERENCES

Eldjarn, L.: Scand. J. clin. Lab. Invest. 1950, 2, 202-208
Strømme, J. H.: Studies on metabolism and biochemical effects of tetraethylthiuram disulphide (disulfiram) and diethyldithiocarbamate. Universitetsforlaget, Oslo, 1965.

J Vessman and A Sundwall (Department of Analytical Chemistry and Department of Pharmacology and Toxicology Research Department of the KABI Group Stockholm Sweden) IDENTIFICATION OF HYDROXYLATED METABOLITES OF TERODILINE IN ANIMAL AND MAN

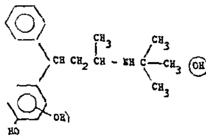
In a prev ous communication terodiline (Bicor) was shown to be extensively metabolized in mouse rat and dog Rather wide species differences were noticed It was therefore important to be able to identify metabolites in human urine

p-hydroxyterodiline which was previously tentatively identified by comparison with the synthetic compound could successfully be gas chromatographed as the trimethylsilylether A compound with the same retention time was found in extracts from β-glucuronidase treated urine from mouse, rat and dog

The identity of the phenol was further established with gas chromatography-masspectrometry Besides this metabolite minor amounts (about 10 %) of two isomeric hydroxylated metabolites were found in mouse urine One of the isomers could be identified as phenol due to the fact that the mass spectrum was very similar to that of the authentic p hydroxy compound The other isomer was an alcohol In rat urine only the p hydroxylated isomer was found In the dog one of the other isomers was present as well

Examination of urine from a patient receiving therapeutic doses *of terodiline* revealed that in man about the same amounts of two hydroxylated isomers were formed one of which was the p-hydroxy isomer They were only present as conjugates with glucuronic ac d

The metabolites are indicated in the figure below



Góran Wahlström (Institute of Pharmacology, University of Uppsala, Sweden): INTERACTION OF ETHANOL AND HEXOBARBITAL IN UNTREATED AND LONG-TERM ETHANOL TREATED MALE RATS.

The sensitivity of hexobarbital was determined in male Sprague Dawley rats. Hexobarbital was infused intravenously at 0.25 mg/kg/sec. The dose needed to obtain a burst suppression of 1 second or more in the EEG was taken as the threshold (Wahlström 1966). In the present experiments ethanol (2 g/kg) was injected i.p. prior to the threshold determination and the blood levels of ethanol measured at the time of the threshold determination. The effect of ethanol on the hexobarbital threshold was determined on the rising and falling part of the blood ethanol curve.

The presence of ethanol reduced the hexobarbital threshold. A blood level of 1.5 - 2.0 mg/ml on the rising part reduced the hexobarbital threshold by approximately 50 %. Corresponding blood levels on the falling part of the concentration curve reduced the threshold by only approximately 25 %. This difference in effects of similar ethanol concentrations indicates that an acute tolerance has been induced. The time lag between brain and blood concentration of ethanol acts in the opposite direction.

Up to blood levels of 2.0 - 2.5 mg/ml of ethanol on the falling part, between 3 and 6 hours after the i p. injection, ethanol and hexobarbital were additive.

In two experiments 10 % w/v ethanol was the sole drinking fluid for four weeks and the rats were only allowed to drink for two separate hours each day. This ensures "intoxication". At the end of the first experiments 1.0 - 2.0 mg/ml of ethanol on the falling part of the blood concentration curve had no influence on the threshold dose (n = 3). In the second experiment there was a decrease in effect of injected ethanol after 4 weeks of treatment compared to results after 2 weeks (n = 8) but a tolerance of such a magnitude as in experiment 1 had not been induced. In both experiments threshold determination without ethanol was uninfluenced by the chronic ethanol treatment.

REFERENCE

Wahlström, G.: Acta pharmacol. et toxicol. 1966, 24, 404-418.

This work has been supported by the Tri-Centennial Fund of the Bank of Sweden.

Birger Winbladh (Research Institute of National Defence, Dept 1, Sundbyberg, Sweden): CHOROID PLEXUS UPTAKE OF ATROPINE AND METHYLATROPINE

Tritium labelled atropine and methylatropine are cleared faster from cerebrospinal fluid (CSF) than inuline (representing bulk clearance) after intraventricular inject

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bulk clearance Since choroid plexus possesses uptake mechanisms for some amines in vitro (Tochino & Schanker 1964; Takemori & Stenwiik 1966) and there seems to be an active transport of choline (Gardiner & Domer 1968) and some other amines (Pollay & Davson 1963) out of CSF we have studied rabbit choroid plexus uptake of T-atropine and T-methylatropine in vitro

Choroid plexus from adult and young rabbits were rapidly excised and bathed in Krebs-Hensenleit solution gassed with carbogen at 37 °C. Tissue and medium concentrations were determined by liquid scintillation.

Both drugs accumulated in choroid plexus against a concentration gradient. The accumulation mechanisms were saturable, sensitive to metabolic inhibitors as dinitrophenol, N-ethylmaleimide and low temperature. Further, accumulation increased with time High voltage electrophoresis revealed no metabolites of the two drugs in tissue or medium. Tissue-medium ratios for adult rabbit plexus were 10 for atropine and 78 for methylatropine after 40 min at a medium concentration of 10^{-4} M. In the newborn rabbit corresponding ratios were 5 5 and 21 respectively.

Uptake of atropine was competitively inhibited by other tertiary amines as morphine and promethazine. Methylatropine uptake on the other hand was inhibited competitively by other quaternary ammonium compounds as choline, hexa- and decamethonium The transport system for tertiary and quaternary amines seem to be independent.

REFERENCES

Gardiner, J E & F R Domer. Arch. int. Pharmacodyn. 1968, 175, 482-496

Pollay M & H Davson: Brain 1963, 86, 137-150.

Takemori A E & M W Stenwiik: J. Pharmacol. exp. Ther. 1966, 154, 586-594

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Erik Änggård, Lars-M. Gunne and Lars-Erik Jönsson (Department of Pharmacology, Karolinska Institutet, 104 01 Stockholm, and Psychiatric Research Center, Ulleråker Hospital, University of Uppsala, Sweden):
RELATIONSHIPS BETWEEN PHARMACOKINETIC AND CLINICAL PARAMETERS IN CHRONIC AMPHETAMINE ABUSE

Eighteen subjects with amphetamine psychosis were studied with respect to urinary and blood amphetamine levels, fluid balance, and psychotic symptoms All psychotic patients were found to be dehydrated on admission *The dehydration amounted up to 7 % of the total body weight* A highly positive correlation was found between urinary pH and the half life ($T_{1/2}$) of plasma amphetamine, with an increase in $T_{1/2}$ of about 7 hours for each increase in unit of urinary pH Patients with alkaline urine had an intense psychosis with a duration of about five days In patients with acidic urine the psychotic symptoms were milder and lasted only for a 2 - 3 days No correlation was found to exist between the degree of psychosis and the levels of drug in plasma

The ratio between labelled metabolites and drug excreted in urine increased for each day after administration of ^{3}H-amphetamine. This indicates a slower elimination rate for the metabolites as compared to the parent drug The proportion between metabolites and amphetamine was higher in the patients with alkaline urine, being over 90 % after the first day

When amphetamine (200 mg i.v.) was given to nonpsychotic, dependent subjects, the peak plasma levels (mean 423 ng/ml) exceeded the mean level observed during the first day in the psychotic patients However, no psychotic symptoms were registered in these subjects Together the results indicate that metabolites of amphetamine may be of importance *in amphetamine psychosis*

Ivar Oye and Asbjorn Langslet (Institute of Pharmacology, University of Oslo, Norway) THE ROLE OF CYCLIC 3 5 -AMP IN THE CARDIAC RESPONSE TO ADRENALINE

The cardiac response to adrenergic stimulation is one of the processes in which cyclic 3 5 -AMP has been allocated a role as intracellular mediator Some workers, however have observed inotropic and chronotropic responses to adrenaline without an increase in the levels of cyclic 3 5'-AMP

In the present work we have examined the effect of exogenous cyclic 3'5'-AMP in the isolated, perfused rat heart, taking advantage of the fact that nucleotides penetrate heart muscle membranes more easily at low temperatures

At 10° both adrenaline and exogenous cyclic 3 5' AMP (5×10^{-5} M) increased phosphorylase a without initiating contractions At 16° both adrenaline and cyclic 3 5 -AMP caused phosphorylase activation, but only adrenaline had inotropic and chronotropic actions Vice versa, low concentrations of adrenaline stimulated contractile activity without activating phosphorylase At temperatures above 22° cyclic 3 5 -AMP did not penetrate the cell membrane to cause phosphorylase activation

The available data suggest that cyclic 3 5 -AMP is not an obligatory mediator of the inotropic and chronotropic action of adrenaline in the rat heart Activation of adenyl cyclase is probably part of a more general alteration of the cell membrane (membrane labilization) when the hormone interacts with its receptor

Erik Änggård, Lars-M. Gunne and Lars-Erik Jönsson (Department of Pharmacology, Karolinska Institutet, 104 01 Stockholm, and Psychiatric Research Center, Ulleråker Hospital, University of Uppsala, Sweden):
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AUTHOR INDEX

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Translated by

ROLF DANIELI, M Sc

Tryckeri Balder AB Stockholm 1971

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Following the ban, other seed dressing agents, such as Voronit®[2] and Neovoronit®[3] have gained increased importance Voronit has been used in Sweden since 1964 Both agents are mixtures containing 2 (2 furyl)benzimidazole (FB)

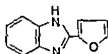


Fig 1 — Structural formula of 2 (2 furyl)benzimidazole (FB) Because of the tautomeric shift in the imidazole nucleus the positions 4 and 7 will be equivalent, as will the positions 5 and 6

Although FB (Fig 1) was first synthesized by Weidenhagen in 1936, the fungicidal properties of the compound were not described until the beginning of the 1950s (Jerchel *et al* 1952) In the years that followed, a number of investigations were carried out, showing that the compound alone or mixed with other compounds is active against a number of genera and species of fungi, in particular the snow mold, *Fusarium nivale* (Frohberger *et al* 1966, Schering AG 1967, Kolk & Ljungberg 1967, Andren 1967, Noddegaard *et al* 1968, Schumann 1968), and that it also possesses anthelmintic properties (Merck & Co 1964, and others)

The use and dissemination of FB in nature prompted a need for knowledge of its toxicology and of methods for determining the compound itself and its metabolites in biological materials Some aspects of the toxicology of FB in the rat have

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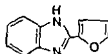


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[3] Neovoronit®: 0.5 % 2-(2-furyl)benzimidazole + 30 % sodium dimethyldithiocarbamate, Farbenfabriken Bayer AG, Leverkusen, Ger.

been treated recently by Kimmerle (unpublished results) An ultraviolet spectroscopic method for determining residues of FB in seed has been reported by Vogeler (1968)

The aims of the present investigation have been as follows

(a) to develop a method for determining FB in biological materials,
(b) to obtain informations about the persistence of FB in the animal organism,
(c) to develop a method for detecting FB metabolites in urine,
(d) to identify and characterize urinary metabolites of FB,
(e) to develop a method for determining FB metabolites in urine

Chapter 1. Detection and determination of 2-(2-furyl)benzimidazole (FB) in biological materials

The general features of a method of analysis for FB in biological materials have been described in a preliminary report by Frank (1966) The present chapter contains a detailed account of this method, which comprises an extraction process, purification and isolation of FB from other substances that may be extracted from the tissues, and the detection and quantitative determination of FB

Part I Development of the analytical method

EXPERIMENTAL

PRINCIPLE

FB is extracted from the organs by means of a mixture of organic solvents applying the lipid extraction method of Folch *et al* (1957) The extract is purified by a liquid liquid partitioning process Remaining extractives are separated by thin layer chromatography (TLC), leading to isolation of FB The confirmation and quantitative assessment of FB in the TLC eluate are carried out by means of ultra violet spectrophotometry

CHEMICALS AND REAGENTS

Chloroform
Ethanol 96 per cent
Methanol
Hexane puriss min 99 per cent
Hydrochloric acid 0 1 M
Buffer solutions covering pH 1—13 ionic strength $\mu=0\ 1$
Extraction mixture chloroform methanol (2 1, by vol)
Partitioning liquids
  Upper phase —chloroform methanol hydrochloric acid (0 1 M), (3 48 47 by vol)
  Lower phase —chloroform methanol hydrochloric acid (0 1 M), (86 14 1 by vol)
Sodium hydrogen carbonate
Sodium sulphate anhydrous
Aluminium oxide GF_{254} (Type E) (E Merck, Darmstadt, Ger)
All reagents were of analytical grade, unless otherwise stated

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Chloroform
Ethanol, 96 per cent
Methanol
Hexane, puriss, min 99 per cent
Hydrochloric acid, 0 1 M
Buffer solutions covering pH 1—13, ionic strength μ=0 1
Extraction mixture, chloroform methanol (2 1, by vol)
Partitioning liquids
  Upper phase —chloroform methanol hydrochloric acid (0 1 M), (3 48 47, by vol)
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Sodium hydrogen carbonate
Sodium sulphate, anhydrous
Aluminium oxide GF_{254} (Type E) (E Merck, Darmstadt, Ger)
All reagents were of analytical grade, unless *otherwise stated*

2 (2 furyl)benzimidazole (FB), m p 283—285° [4], was a gift from Farbenfabriken Bayer AG, Leverkusen, Ger (Lit, m p 285—286°, Weidenhagen 1936) Freshly prepared solution of FB in ethanol, containing 1 μg per μl was used as standard solution

EQUIPMENT AND APPARATUS

High speed blender and homogenizer (MSE, London, Brit)
Rotary evaporator (Stålprodukter, Uppsala, Sweden)
Recording UV spectrophotometer (Beckman Model DK 2), 10 mm quartz cells
pH meter (Type PHM 28, Radiometer, Copenhagen, Denmark)
TLC spreading apparatus (Camag, Muttenz, Switzerland)
Glass plates 10×20 cm
Developing tank
UV detection lamp, 254/366 (Camag)
Glass wool, fibres about 1 μm (Bilsom International AB, Billesholm, Sweden)
Glass filter tubes, medium porosity

ANALYTICAL PROCEDURE

Extraction of biological materials

Animal tissues and ingesta — Tissue material or ingesta (1 g) are homogenized during 5 min with extraction mixture (18 ml) in a high speed blender The mixture thus obtained is filtered through a glass filter directly into a separating funnel applying slight suction The blender cup and filter are rinsed with extraction mixture (2 ml)

Blood — Plasma or serum (1 ml) is added dropwise to extraction liquid (18 ml) in an Erlenmeyer flask under continuous and efficient agitation The mixture is filtered directly into a separating funnel through glass wool which removes the precipitated proteins The flask and protein precipitate on the glass wool are finally washed with extraction liquid (2 ml)

Purification of the extract by liquid-liquid partitioning

Hydrochloric acid (0 1 M, 4 ml) is added to an extract (20 ml) obtained according to ' Extraction of biological materials the mixture being shaken carefully for 3 min in a separating funnel Separation of the phases occurs rapidly when the shaking is discontinued The lower phase is drawn off and extracted twice with 'upper phase (8 ml)

The combined upper phase is washed with lower phase (2 ml) which is subsequently removed, the combined 'upper phase is then neutralized with solid sodium hydrogen carbonate (about 0 5 g) and water (16 ml) is added The

[4] The melting point was determined on a melting point microscope with a Kofler micro hot stage (C Reichert, Vienna Austria) All temperatures are given in degrees centigrade

solution is extracted with chloroform first once with 20 ml and then twice using 10 ml each time the duration of the individual extractions being 3 *min*

The combined chloroform extracts are dried over anhydrous sodium sulphate, filtered through glass wool in a round bottomed flask. The sodium sulphate and glass wool are washed with *chloroform (a few ml), which is subsequently* evaporated together with the chloroform extracts in a rotary evaporator on a water bath at room temperature and under reduced pressure

Thin layer chromatographic (TLC) separation of FB

Aluminium oxide thin layer plates (chromatoplates) were prepared with a wet-layer thickness of 0 3 mm After the plates had been dried in the air they were activated by heating (1 h, 105°) and stored in a desiccator until used

The evaporated chloroform extract was transferred quantitatively to the plate by repeated washings of the evaporating flask with chloroform For each washing 300 μl of chloroform was used The plate was also provided with an FB reference solution corresponding to 2—5 μg of FB The plate was developed in a glass jar containing a chloroform hexane mixture (1 1, by vol) as developer Equilibration of the atmosphere of the developing tank was achieved by lining the interior surfaces of the tank with filter paper that was moistened with the liquid developer

Satisfactory separation of FB was obtained after the liquid front had migrated 10 cm Improved separation was achieved when the liquid front was allowed to migrate a distance of 10 cm twice, with the plate being dried between developments

On examining the plate in ultraviolet light (254 nm)[5] FB appears as a dark blue spot against a yellow fluorescent background The method permits quantities of 0 2—0 3 μg to be detected

Elution and quantitative evaluation by means of UV spectrophotometry

The adsorbent is scraped off from relevant areas of the plate, transferred quantitatively to small glass filter tubes and the compound is eluted with ethanol (2 ml) in small portions The volume is adjusted to exactly 2 ml and the absorbance of the solutions is measured on a recording spectrophotometer in quartz cells (10 mm) over the 250—360 nm range using ethanol as a blank. For each solution the corrected absorbance A_{corr} is calculated according to the following formulae

$$\Delta A = \Delta y + A_2 \qquad \Delta y = 0\,568 \left[(A_1 - A_2) - \frac{(A_0 - A_2)}{k}\right]$$

$$A_{corr} = A_0 - \Delta A \qquad \text{FB } \mu g/ml = \frac{A_{corr}}{\epsilon}$$

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[5] Because of the sensitivity of FB to light all work involving FB and its derivatives should be carried out under adequate light protection

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[4] The melting point was determined on a melting point microscope with a Kofler micro hot stage (C Reichert, Vienna, Austria) All temperatures are given in degrees centigrade

In the quantitative analysis of certain benzimidazole derivatives such as thiabendazole (TBZ)[6] and benomyl[7] use has been made of fluorimetry (Tocco *et al* 1965 Pease & Gardiner 1969) By applying this technique it is possible to achieve a slightly greater sensitivity than by UV photometry In the case of TBZ it has also been possible to simplify the technique of purifying the crude extract, owing to the specificity of the fluorescence at suitable wavelengths From a comparison of the values (Pease & Gardiner 1969) it is obvious however that FB shows the lowest relative intensity in fluorimetric analysis of TBZ FB and benomyl

CHROMATOGRAPHIC SEPARATION AND ELUTION

For the purpose of TLC separation of FB investigations of various solvent systems have proved a mixture of equal volumes of hexane and chloroform on aluminium oxide to be the most adequate The spots obtained in this way are round and well defined with R_f values of 0 2—0 3 Contaminants carried on from the extraction procedure separate well or are retarded efficiently With high concentrations of extractive compounds present, satisfactory results can be obtained by repeated chromatography

The adsorbent proved to possess high tolerance towards extractives but owing to the extraction and purification technique only minor quantities of UV absorbing compounds were carried on With tissue extracts purified according to this procedure, aliquots equivalent to as much as 3 g of sample could be applied without overloading the chromatogram Investigations have shown that this amount may be increased up to 5 g in certain cases (liver)

EXTRACTION OF BIOLOGICAL MATERIALS PURIFICATION OF FB BY LIQUID LIQUID PARTITIONING

At the pH values of current interest in biolog cal materials (tissues blood plasma) FB appears to be uncharged and of a lipoph lic nature For this reason an extract on technique for total lipids (Folch *et al* 1957) has been applied This method has advantages such as very efficient extraction properties and the absence of annoying emulsion formation The extracting solvent (chloroform methanol) extracts all lipids and dehydrates the tissue almost completely The volume and composition of the solvent are so adjusted as to allow the liquid to remain homo geneous even after the extraction On the addition of water (acidified with hydrochloric acid in this case) the phases separate despite the high lipid content in the lower phase without giving rise to emulsification The compos tion of the phases is well defined By preparing the upper phase and lower phase sepa rately the liquid liquid partitioning of FB can be accomplished under constant cond tions *i e* without changes in the composition of the liquid phases The process does not involve any emuls fication problems

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[6] 2 (4-th azolyl)benzim dazole

methyl 1 (butylcarbamoyl) 2 benzim dazolecarbamate

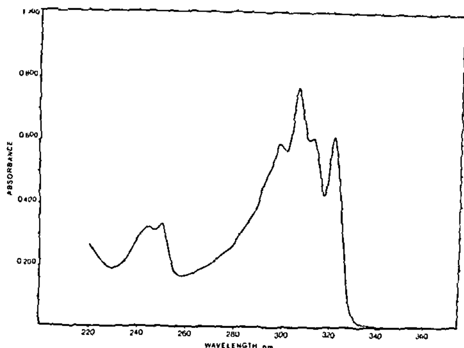


Fig 2 — UV spectrum of FB in ethanol (96 %), 5 µg/ml

Here A_1, A_0 and A_2 are the absorbances $\times 10^3$ at wavelengths 278 307 and 345 nm, respectively ΔA is the total correction, Δy is the part of the correction related to irrelevant absorption according to Vandenbelt & Shearer (1962)

$0.568 = \frac{345-307}{345-278}$ $\qquad k = \frac{A_0}{A_1}$ is a constant of the pure substance, in the case of FB k = 3 1 The FB content in the sample is determined by means of the experimentally established extinction coefficient "ε = 152 3, using the reference compound (*cf* "Chemicals and Reagents)

COMMENTS ON THE METHOD

UV SPECTROPHOTOMETRIC DETERMINATION

FB has a large molar extinction coefficient at its greatest absorption maximum, 307 nm in ethanol, log ε = 4 45 (*cf* Table 15, p 93) Thus, UV photometry is well suited to quantitative determination of FB and has also been used for the evaluation of residues of FB in seed (Vogeler 1968) —At contents of only 0 8—1 0 µg for each sample the absorbance in the sample solution (ethanol) is 0 060—0 076 applying the procedure described At still lower contents the un certainty of the determinations is greater, owing to irrelevant absorption Although it is still possible to detect and identify FB even at contents below the values mentioned above by utilizing the characteristic UV curve of the substance (*Fig* 2), there will be considerable errors in the quantitative evaluation

Table 1 — Standard deviation (SD) and recovery for different amounts of FB following thin layer chromatography and elution

µg FB added	Number of determinations	µg FB found (range)	µg FB (mean)	Standard deviation µg	Recovery %
0 47	5	0 41—0 46	0 438	0 022	93 2±5 0
4 72	5	4 37—4 56	4 472	0 080	94 7±1 8

B *Solvent system chloroform water methanol* The distribution of FB between upper phase and 'lower phase (*cf* 'Chemicals and Reagents) was investigated at pH 8 and pH 1 under the same conditions as in A (above)

RESULTS

FB is ionized in strongly alkaline as well as in strongly acid solutions It is, consequently possible to extract FB from an organic solvent by means of an aqueous solution with a pH <2 or >12, and to extract it from an aqueous solution of pH 5—11 by means of an organic solvent

Solvent system A The percentage distribution of FB between chloroform and water as a function of the pH value is shown in Fig 3 It can be seen from the figure that within the pH range 5—9, 98 per cent of FB is present in the chloroform phase and that 96 per cent of FB is present in the aqueous solution at pH <2

Solvent system B In this case 78 per cent of the FB appeared in the "upper phase at pH 1 and 88 per cent in the 'lower phase at pH 8 Hence the presence of methanol to some extent influences the distribution of FB between the liquid phases

CONCLUSIONS

The recovery during the liquid liquid partitioning according to the procedure can be calculated to be 91—92 per cent whereas the overall recovery, including the losses associated with the TLC process can be estimated to be 85—86 per cent

RECOVERY OF FB AFTER ADDITION TO LIVER EXTRACT AND TO BLOOD PLASMA

EXPERIMENTAL

A Beef liver extract was prepared according to the standard procedure, various quantities of FB (1 11—4 80 µg) then being added to 60 ml portions of the liver extract corresponding to 3 g of liver The samples were subsequently analyzed

B FB was added to bovine serum to yield serum concentrations of FB corre

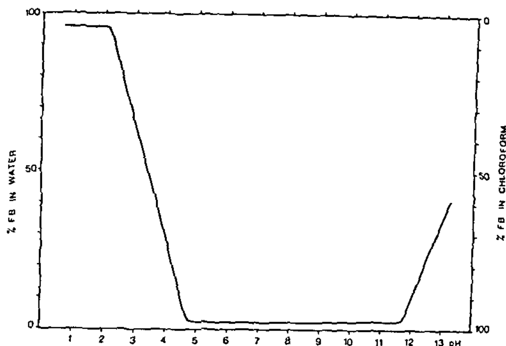


Fig 3 — Partition of FB in chloroform and water at different pH values

Part II. Studies of method reliability

Standard statistical methods were applied during the calculations as indicated below (Youden 1951)

RECOVERY OF TEST COMPOUND AFTER TLC

Table 1 shows the recovery of FB after TLC followed by elution according to the procedure Two series of five samples, each containing 0 47 or 4 72 μg of FB have been determined As can be seen the recovery was 93—95 per cent The coefficient of variation increased when determining smaller quantities

DISTRIBUTION OF FB IN LIQUID-LIQUID PARTITIONING

EXPERIMENTAL

A Solvent system chloroform water FB (100 μg) in a buffer (20 ml) was extracted (10 min) with chloroform (20 ml) in a separating funnel The pH value of the buffers varied from 1 to 13, with individual steps of one pH unit The ionic strength was $\mu = 0\ 1$ in all solutions The absorbance of the buffer solutions was registered by a recording UV spectrophotometer before and after chloroform extraction The amount of FB extracted by the chloroform solution was calculated by comparing the absorbance of the buffer solutions at the maximum of absorption before and after the extraction The current buffer solution was used as a blank and treated in exactly the same way as the sample liquid

Table 4 — Tissue levels and total amount of FB recovered from stomach ingesta in a rabbit given orally 100 mg FB per kg body weight and killed after 30 min

Liver FB µg/g	13 33	13 63		
Kidney FB µg/g	9 46	9 62		
Blood plasma FB µg/ml	8 39	8 46	8 48	9 01
Stomach ingesta				
total amount of FB mg	97	99		
per cent of dose	46			

determinations underlying this part of the investigation is somewhat greater than the value obtained on analysis of FB in liver extract (78 per cent)

DETERMINATION IN TISSUES OF RABBIT GIVEN FB ORALLY

EXPERIMENTAL

The test animal used for this experiment was a white male albino rabbit, three months old, weighing 2 150 g The animal was maintained on a standard diet consisting of pellets hay and water The pellets were supplied by AB Ewos, Sodertalje Sweden

FB was dissolved in the theoretical quantity of hydrochloric acid (0 1 M), the final volume being adjusted with water to give 10 mg FB per ml The pH of the solution was 2 8 The solution was administered to the animal by stomach tube in a quantity corresponding to 100 mg FB per kg body weight

The rabbit was killed after 30 min by an intravenous injection of a lethal dose of Mebumal® (pentobarbital) Blood was obtained by cardiac puncture by means of a heparinized syringe Liver, kidneys and stomach were removed for analysis The biological materials were stored at —20° until analyzed

RESULTS AND CONCLUSIONS

As can be seen from Table 4, duplicate samples taken from the liver kidneys and stomach contens were in good agreement Four analyses of blood plasma showed good reproducibility (mean 8 6 µg FB per ml plasma) The experimental results prove this method of analysis to be adequate for determining FB in various types of biological material

SUMMARY

The present work describes a method of isolation and determination of 2 (2-furyl)benzimidazole (FB) in biological materials of animal origin The sample is extracted with an organic solvent mixture FB is separated from extractives by partition between solvent and aqueous phase at pH 1 in the presence of methanol

Table 2 — Regression calculation for FB added in various amounts to liver extract

Number of analyses N	16
Regression coefficient, b	0 778 ± 0 055 P = 5 %
Intercept a, (μg)	0 162
S D of any individual analysis, s, (μg)	0 146
S D of intercept, s(a), (μg)	0 083
S D of slope of regression line, s(b)	0 027
S D of slope in per cent	3 54
t test for passage through origin, t(a)	1 95 accept

sponding to 0 305 and 3 05 μg/ml One ml samples were analyzed and the concentrations were calculated according to the standard procedure

RESULTS

A Table 2 shows the result obtained from 16 determinations of FB concentrations in *liver* extract, According to the regression coefficient, the recovery (78 per cent) is slightly below the theoretical value (*t e*, 86 per cent according to calculations of the recovery at TLC and liquid liquid partitioning) The S D for any single observation in Table 2 refers to the average deviation for each analysis within the region measured It has been demonstrated earlier, however, in determining the recovery at TLC (Table 1) that the coefficient of variation increases at smaller amounts of FB Because of this increase the error affecting determinations of very small amounts (<1 μg FB per sample) will be greater than otherwise Other magnitudes as well, calculated in Table 2 as intercept and S D of intercept, contribute to the uncertainty in determining such small amounts

B The results obtained when determining FB added to bovine *serum* are summarized in Table 3 It should be pointed out that there were some difficulties in estimating such low amounts of FB in serum as 0 31 μg/ml The absorbance is so low that the intrinsic error of the instrument begins to be noticeable in addition to the effects of contaminants and irrelevant absorption The S D is 4—5 per cent and the recovery (84—87 per cent), as obtained from the small number of

Table 3 — Standard deviation and recovery for different amounts of FB added to serum

μg FB added	Number of determinations	μg FB found (range)	μg FB (mean)	Standard deviation μg	Recovery %
0 31	4	0 26—0 28	0 27	0 01	87 1 ± 5
3 05	6	2 42—2 71	2 57	0 107	84 3 ± 4 2

Chapter 2. On the gastro-intestinal absorption and the persistens of FB in dog and rat

The present chapter demonstrates the applicability of the analytical method presented in Chapter 1 and describes the degradation of FB in the animal organism.

EXPERIMENTAL

ANIMALS AND PROCEDURE

Four Sprague Dawley female albino rats (AB Anticimex, Sollentuna, Sweden), weighing 135—145 g each, were used in this investigation The rats were fed a complete pelleted rat diet (pellets no 213, 'Breeder chow) and water *ad libitum* The pellets which were supplied by AB Anticimex, had the following composition (per 100 g) as stated by the manufacturer crude protein, 20—22 g, crude fat, 4 5—5 5 g vegetable fibres, 11—13 g and minerals, vitamins and trace elements

One male mongrel dog 8—9 months old and weighing 8 5 kg was used in the experiment The dog was fed 0 5 kg/day of 'Doggy dog feed (Vårgårda, Sweden) The composition (per 100 g) was given by the manufacturer as animal protein 20 g animal fat 8 g, undigestible vegetable fibres, 2 g and minerals vitamins and trace elements

An aqueous solution of FB containing 10 mg/ml as described on p 17, was administered by means of a disposable syringe to which a blunt needle was attached at a dose of 100 mg FB per kg body weight to each of the four rats The animals were killed by intraperitoneal injection of Mebumal® (pentobarbital) 15, 30, 60, and 120 min respectively after FB administration Blood samples were withdrawn by cardiac puncture using a heparinized syringe, in connection with the killing The plasma was separated by centrifugation and stored at —20° until analyzed The liver was also removed and stored at —20° until analyzed

The dog was starved during 15 h prior to the FB administration and was not fed during the experiment, but had access to water *ad libitum* The dog received 56 4 mg FB per kg body weight in a gelatin capsule Blood samples were taken by means of a heparinized syringe from the brachial vein at various intervals (*cf* Fig 5) after the FB administration The plasma was separated by centrifugation and stored at —20° until analyzed To enable the urine to be collected, the dog was kept in a metabolism cage throughout the duration of the experiment (24 h)

ANALYSIS

Liver and plasma were analyzed according to the method described on p 10 Duplicate determinations were always carried out on the liver material, and as a

The detection limit of FB in the thin layer chromatography stage is 0 2—0 3 μg Ultraviolet spectrophotometry permits determination down to 0 8—1 0 μg per sample

The method was found to give satisfactory results at determination on tissues from experimental animals which have been given FB orally

Following thin layer chromatography and elution from the adsorbent, FB is determined quantitatively by ultraviolet spectrophotometry

In analyses of liver and serum to which known amounts (0 3—4 8 μg) of FB had been added, the recovery was 75—90 per cent, with coefficient of variation corresponding to a maximum of ±6 per cent

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ANALYSIS

Liver and plasma were analyzed according to the method described on p 10 Duplicate determinations were always carried out on the liver material, and as a

rule also on the dog plasma The modest amount of rat plasma available only permitted single determinations to be made

Urine (25 ml) was extracted with chloroform (1×25 ml and 2×10 ml) The chloroform extracts were combined and washed with phosphate buffer (pH 7, μ=0 1, 5 ml) and were then extracted with hydrochloric acid (0 1 M, 2×15 ml) The aqueous solution was neutralized with solid sodium hydrogen carbonate (0 5 g) and extracted with chloroform (1×20 ml and 2×10 ml) The subsequent steps of the analysis coincided with the description given on p 10

RESULTS

Rats — 15 min after FB had been given, the concentrations of FB in blood plasma and liver were 21 μg/ml and 86 μg/g, respectively There was then a rapid drop in the concentration of FB as can be seen in Fig 4 After 2 h the FB con centration in the plasma was <1 μg/ml and 2—3 μg/g in the liver The ratio of the plasma and liver concentrations remained comparatively constant, about 1 4 during the experiment

Dog — The maximum blood plasma concentration of FB, 7—8 μg/ml, was reached after 1 5—2 h As can be seen from Fig 5 the elimination of FB from the plasma was rapid once the peak level had been reached After 4 h the plasma level of FB was only 0 1 μg/ml, and after 5 h it was no longer possible to trace the compound

No FB could be detected in the urine

DISCUSSION

The results of the present investigation in rat and dog indicate a rapid absorp tion of FB from the alimentary tract in both animal species Judging from the experiments the difference of maximum plasma levels in time, 15 min and 2 h in rat and dog, respectively, may depend both on the difference in animal species used as well as on the difference in physico chemical form of FB administered Similar results have previously been noted in the rabbit experiment (*cf* Table 4 p 17)

In the rat and rabbit experiments the administered FB was dissolved in dilute hydrochloric acid Such a treatment of the test compound will probably facilitate absorption from the alimentary tract

When FB was given as free base, which was the case in the experiment on the dog the compound may dissolve in the acid environment of the stomach Obviously this process, which was accompanied by digestion of the gelatin capsule has to take a certain time This might be one reason why the absorption was delayed in com parison with the rat and rabbit where the dissolution of the compound was accomplished before the compound entered the intestinal tract

The disappearance of FB from the blood plasma follows the same pattern in the rat and dog (*cf* Figs 4 and 5) In both species the plasma level dropped to

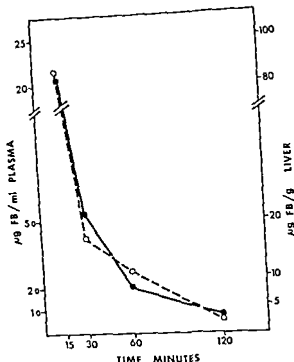


Fig 4 — Blood plasma and liver levels of FB in rats after a single oral dose of FB dissolved in dilute hydrochloric acid (100 mg FB per kg body weight) Each point corresponds to one animal •——• Blood plasma o- -o liver values

<1 μg/ml within 2 h after the peak plasma levels had been observed Thiabendazole which is closely related to FB chemically and structurally, has been reported to behave in a similar manner in the dog (Tocco *et al* 1966)

In the rats the concentration of FB in the liver was about four times higher than in the blood plasma at all times investigated The plasma level obviously is in equilibrium with the concentrations in the liver Considering that the concentration in the liver decreases at the same rate as the plasma level, it may be concluded that the equilibrium is rapidly displaced towards the formation of more polar products *i e* that the biotransformation of FB is a fast process

On analysis of dog urine collected during 24 h no FB was detected This observation is in agreement with later investigations of urine samples of dogs and other animal species after FB administration In no case could any FB be detected in the urine (*cf* Chapter 3 p 36 and Chapter 4 p 44)

The present investigation suggests that probably no residual content of FB will be found in animals (game) that may have eaten FB in quantities corresponding to the amounts that are used under field scale conditions (60 mg FB per kg seed) The method of analysis presented in Chapter 1, however, has proved its usefulness in quantitative determination of FB in biological materials

rule also on the dog plasma The modest amount of rat plasma available only permitted single determinations to be made

Urine (25 ml) was extracted with chloroform (1×25 ml and 2×10 ml) The chloroform extracts were combined and washed with phosphate buffer (pH 7, μ=0 1, 5 ml) and were then extracted with hydrochloric acid (0 1 M, 2×15 ml) The aqueous solution was neutralized with solid sodium hydrogen carbonate (0 5 g) and extracted with chloroform (1×20 ml and 2×10 ml) The subsequent steps of the analysis coincided with the description given on p 10

RESULTS

Rats — 15 min after FB had been given, the concentrations of FB in blood plasma and liver were 21 μg/ml and 86 μg/g, respectively There was then a rapid drop in the concentration of FB as can be seen in Fig 4 After 2 h the FB con centration in the plasma was <1 μg/ml and 2—3 μg/g in the liver The ratio of the plasma and liver concentrations remained comparatively constant, about 1 4, during the experiment

Dog — The maximum blood plasma concentration of FB, 7—8 μg/ml was reached after 1 5—2 h As can be seen from Fig 5 the elimination of FB from the plasma was rapid once the peak level had been reached After 4 h the plasma level of FB was only 0 1 μg/ml, and after 5 h it was no longer possible to trace the compound

No FB could be detected in the urine

DISCUSSION

The results of the present investigation in rat and dog indicate a rapid absorp tion of FB from the alimentary tract in both animal species Judging from the experiments the difference of maximum plasma levels in time, 15 min and 2 h in rat and dog, respectively may depend both on the difference in animal species used as well as on the difference in physico chemical form of FB administered Similar results have previously been noted in the rabbit experiment (*cf* Table 4 p 17)

In the rat and rabbit experiments the administered FB was dissolved in dilute hydrochloric acid Such a treatment of the test compound will probably facilitate absorption from the alimentary tract

When FB was given as free base, which was the case in the experiment on the dog the compound may dissolve in the acid environment of the stomach Obviously this process, which was accompanied by digestion of the gelatin capsule has to take a certain time This might be one reason why the absorption was delayed in com parison with the rat and rabbit where the dissolution of the compound was accomplished before the compound entered the intestinal tract

The disappearance of FB from the blood plasma follows the same pattern in the rat and dog (*cf* Figs 4 and 5) In both species the plasma level dropped to

Chapter 3. Detection and chromatographic separation of urinary metabolites of FB

Investigations on dog and rat suggested that the absorption of FB from the alimentary tract in these species is a rapid process (Chapter 2) In rat and dog maximum blood plasma levels were reached within 15 min and 2 h, respectively, after FB administration A few hours later no FB could be detected in blood from dog, rat and in the liver from rat In dog urine FB could not be detected at all These observations seem to indicate that FB undergoes rapid metabolism. To further elucidate this matter the occurrence of FB metabolites in horse, goat, dog, rabbit, and rat urine was investigated by a technique including column chromatography and spectrophotometry

EXPERIMENTAL

PRINCIPLE

Samples of urine were chromatographed on a Sephadex column The UV absorption of the column eluates was recorded continuously at 280 nm The eluates were divided by a fraction collector into small fractions whose UV spectra were examined over the range 220—360 nm The investigation was carried out on urine samples from the experimental animals before and after FB administration Comparisons of the UV data obtained from these urine samples disclosed the presence of metabolites

ANIMALS AND PROCEDURE

Species, sex, age, and weight of the experimental animals can be seen from Table 5

Horse — The thoroughbred horse was fed daily with hay (7 9 kg), oats (2 2 kg) wheat bran (0 7 kg), carrots (1 0 kg), and water *ad libitum*

Goat — The animal was fed daily with a barley and oats concentrate (0 5 kg), hay and water *ad libitum*

Dogs — Feeding and feed composition as stated previously (p 19)

Rabbit — Diet as indicated previously (p 17)

Rats — Sprague-Dawley rats were obtained from AB Anticimex. Diet as indicated previously under 'Rats' (p 19)

The dogs, rabbit and rats were starved for 15 h before the FB administration and were not fed during the first day of the experiment The dogs were starved

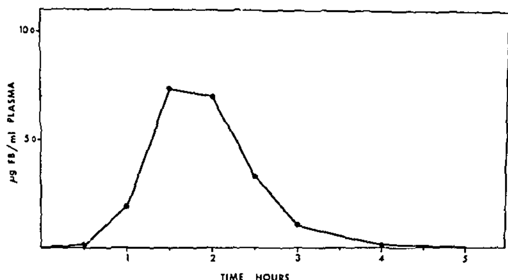


Fig 5 — Blood plasma levels of FB in dog after a single oral dose of FB as free base (56 4 mg FB per kg body weight) The dog was starved 15 h before dosing

SUMMARY

Following oral administration to rats of FB dissolved in dilute hydrochloric acid the absorption from the alimentary tract was rapid The maximum concentration of FB in plasma was observed 15 min after the administration of the compound In the dog, FB given as free base was absorbed at a somewhat slower rate The maximum concentration in the blood plasma occurred 1 5—2 h after the ad ministration

In the liver of rat the concentrations of FB were about four times higher than in plasma at the corresponding time of sampling

The elimination of FB from the plasma was rapid in both dog and rats the maximum plasma level decreasing to <1 µg/ml within 2 h

No FB could be detected in dog urine

The experimental results indicate that the biotransformation of FB is a rapid process, and that hence FB in organs from animals such as game that may have ingested the compound under field scale conditions is not likely to be traced

The method of analysis developed for quantitative determination of FB in bio logical materials proved satisfactory

Rabbit — FB (195 mg) was suspended in water (2 ml) The suspension was administered by stomach tube and was washed down with water (10—15 ml).

Rats — FB was dissolved in dilute hydrochloric acid as previously described on p 17 The test solution was given by means of a disposable syringe to which a blunt needle was attached

Collection of urine samples For collection of urine the animals (except the horse, *vide infra*) were placed in metabolism cages In the rat tests FB was given simultaneously to three animals, which were then placed together in a metabolism cage for collecting the urine The samples were collected during periods of 24 h, as shown in Table 5 The collector for the horse urine consisted of a rubber bag suspended in a strap and connected to a polythene sampling bottle. The horse urine was collected during a period of 18 h

For comparison purposes urine samples were collected from untreated animals during a period of 24 h a few days before the experiments

The pH value of the urine samples was determined with pH meter (p 10) All urine samples were stored at —20° until analyzed

COLUMN CHROMATOGRAPHIC FRACTIONATION OF URINE

A glass column (inner diameter, 10 8 mm) was packed with preswollen Sephadex G 25 gel, fine (Pharmacia, Uppsala, Sweden) to a height of 90 cm

The Sephadex powder was poured into an ammonium hydrogen carbonate buffer (0 1 M, pH 8), containing *n* butanol (1 % V/V) to prevent microbial growth The gel was allowed to swell for 24 h in the buffer The swollen gel was washed several times with the buffer and the fine particles were decanted The column was packed with the gel slurry by sedimentation and was equilibrated with the buffer by 4—5 days percolation

Samples of urine (1—2 ml) were introduced at the top of the column The glass surface inside the column top was rinsed carefully with the buffer (1—2 ml in small portions) and the buffer solution was allowed to wash the sample down into the column The column was filled up with buffer and was connected by a rubber stopcock and polythene tubing to an LKB 4912A peristaltic pump, which maintained a constant flow of 2—3 ml h 1 cm 2 through the column The transmission of the eluate was recorded continuously at 280 nm by means of an LKB 8300 Uvicord II ultraviolet absorptiometer, using a 0 3 cm flow cell The column effluent was collected in fractions (2—3 ml), using a time-controlled automatic fraction collector The separation was considered to be ended when the UV monitor again recorded the constant base line (and no more UV light absorbing compounds could be detected in the effluent by UV spectrophotometry) The separation process usually went on for 5—6 days

The distribution coefficients (K_D) (Gelotte 1960, Determann 1967) were calculated from the equation $K_D = (V_e - V_o)/V_i$, where V_e = elution volume, V_o = void volume of column (35 1 ml), V_i = internal aqueous volume of the gel (38 2 ml

Table 5 — Data on experimental animals and doses of FB administered

Species	Age	Weight kg	Dose mg/kg	Urine collection days after dosing	Total volume of urine ml	pH
horse gelding	14 yrs	570	35 1	18 h	6200	7 3
goat female	—	34	82 4	1st 2nd	245 210	8 5 8 7
rabbit dwarf male	8—9 mts	2 0	97 5	1st 2nd	231 260	8 2 8 3
rats males	—	0 165 0 178 0 226	100 0 100 0 100 0	1st 2nd	34 26	6 7 8 6
dog mongrel male	8—9 mts	11 2	100 0	1st	134	6 8
beagle male	5 mts	7 4	71 2	1st	78	6 0
male	6 mts	7 8	65 2	1st 2nd	150 153	6 0 5 5
male	6 mts	7 5	80 1	1st 2nd	122 137	7 0 7 8
male	6 mts	7 3	91 8	1st 2nd	180 57	6 3 5 7
male	7 mts	7 8	168 6	1st 2nd	131 73	5 7 6 0

on the second day as well This was done in order to avoid contamination of the urine by feed debris falling down All animals were offered water *ad libitum* throughout the experiment

Administration of test compound The single oral doses in mg FB per kg body weight are shown in Table 5

Horse — FB (20 g) was given in the form of a tablet (FB 20 g, Radix glycyrrhizae, 8 g, Constit, 24 g)

Goat — FB (2 8 g) was dissolved in hydrochloric acid (0 5 M 32 ml) A stomach tube was used to bring the test solution into the rumen

Dogs — The compound was given orally in gelatin capsules

Table 7 — Thin layer chromatography R_f values and colour reactions of the metabolites observed in the present investigation.

The following solvent systems were employed

I chloroform methanol ammonia (25 %) (2 2 1 by vol)
II ethyl acetate methanol water (8 3 2 by vol)
III benzene methanol glacial acetic acid (11 4 1, by vol)
IV isobutanol saturated with water

Spray reagents
NR naphthoresorcinol reagent (Stahl 1962)
FC Folin Ciocalteu reagent (Elliott 1959)

Metabolite	Solvent I	Solvent II	Solvent III	Solvent IV	Colour reaction with NR	FC
A	0 45	0 35	0 32	0 09	none	none
B	0 35	0 23	0 05	0 00	reddish brown	none
C	0 65	0 56		0 24	none	none
D	0 59	0 55		0 26	?	blue
E	0 62	0 50	0 20	0 18	none	none
F_1	0 37	0 33		0 10	none	blue
(F)	(0 42)	(0 53)		(0 23)	?	none
Z (=HOFB)	0 88	0 74	0 38	0 75	none	blue

mentioned in Table 7 and air drying the chromatograms were studied in UV light at 254 and 366 nm using a Camag UV lamp, and absorbing and/or fluorescent spots were outlined

In certain cases the plates were sprayed with naphthoresorcinol reagent (Stahl 1962) for detection of glucuronides and with Folin Ciocalteu reagent (Elliott 1959) for detection of free phenolic hydroxyl groups

As a preliminary to UV examination, the compounds in relevant spots on the chromatograms were eluted in small, medium porosity glass-filter tubes, using ethanol The solvent systems (R_f values and colour reactions of metabolites observed) are given in Table 7

RESULTS AND DISCUSSION

OBSERVATIONS ON EFFECTS OF FB IN EXPERIMENTAL ANIMALS

Among the experimental animals used in the present investigation only the dogs have displayed any deviation from their normal behaviour, following administration of single oral doses of FB In about one-third of the dogs, vomiting occurred some 2 h after FB was given This interval coincides with the point at which the maximum plasma concentration was observed in the dog (*cf* Fig 5, p 22) No correlation could be established between the size of the doses and the vomiting frequency It seems as though a certain individual sensitivity may also have been

Table 6 — Elution data from a Sephadex G 25 gel column (1 1 cm×90 cm) from which the distribution coefficients (K_D) of metabolites, FB, hippuric acid, and uric acid were calculated

Metabolite	Elution volume V_e ml	K_D
A	111	2 0
B	191	4 1
C	211	4 6
D	222	4 9
E	248	5 5
F	267	6 0
Z (HOFB)	385	9 1
hippuric acid	92	1 5
uric acid	132	2 5
FB	298	6 9

calcd) V_i was calculated from the equation $V_i = (V_t - V_o)\ W_r\ d\ /\ (1 + W_r)$, where V_t=total volume of column (82 4 ml), W_r=water regain of the gel and d=wet density of the swollen gel particles (d and W_r values were stated by the manu facturer as d=1 13, W_r=2 5±0 2) The elution volumes of the metabolites were estimated by checking the UV absorption and measuring the corresponding volume of the effluent The void volume was considered to be identical with the elution volume of Blue Dextran 2000 (Pharmacia) (Table 6 contains the K_D values and elution data for the metabolites, FB, hippuric acid and uric acid)

UV SPECTROPHOTOMETRIC INVESTIGATION OF COLUMN ELUATES

The fractions obtained by the column chromatographic separation of the urine samples were examined by UV spectrophotometry The spectra were obtained on a Beckman Model DK 2 recording spectrophotometer, using 10 mm quartz cells Measurements were run in the 220—360 nm range against ammonium hydrogen carbonate buffer (0 1 M) as blank Where required, dilutions were made using the same buffer

TLC OF COLUMN ELUATES

In TLC the same equipment was used as previously mentioned for TLC of FB (Chapter 1) The chromatoplates were prepared with Silica gel GF_{254} (E Merck) The wet layer thickness was 0 3 mm After the plates had dried in the air they were activated by heating for 1 h at 105° and then stored in a desiccator until used

The eluates or the pooled and concentrated (at reduced pressure, using a rotary evaporator) fractions from the column chromatographic separation of urines were transferred to thin layer plates After development with any of the solvent systems

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E	0 62	0 50	0 70	0 18	none	none
F_1	0 37	0 33		0 10	none	blue
(F_2)	(0 42)	(0 53)		(0 23)	?	none
Z (=HOFB)	0 88	0 74	0 38	0 75	none	blue

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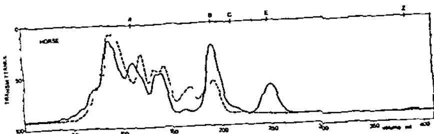


Fig 6 — Chromatographic elution pattern of one ml of horse urine (18 h) from a Sephadex G 25 column (1 1 cm×90 cm), using ammonium hydrogen carbonate buffer (0 1 M, pH 8) at a flow rate of 2 4 ml/h—*After a single* oral dose of 35 1 mg FB per kg body weight Normal urine The curves were measured at 280 nm

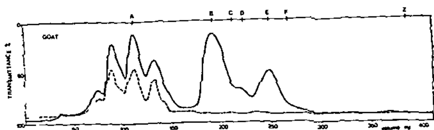


Fig 7 — Chromatographic elution pattern of one ml of goat urine (24 h) at an elution rate of 2 4 ml/h —After administration of a single oral dose of 82 4 mg FB per kg body weight Normal urine (Otherwise as in Fig 6)

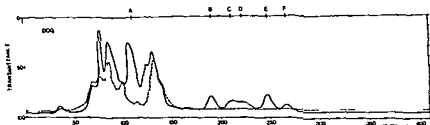


Fig 8 — Chromatographic elution pattern of one ml of dog urine (24 h) at an elution rate of 2 4 ml/h — After administration of a single oral dose of 71 2 mg FB per kg body weight Normal urine (Otherwise as in Fig 6)

The UV spectra show similarities with UV spectra of benzimidazole or of its derivatives A working knowledge of the spectral properties of benzimidazole compounds thus facilitates the detection and identification of FB metabolites with an intact benzimidazole skeleton Benzimidazole derivatives are characterized by strong UV absorption and often by typical UV spectra (Leandri *et al* 1955, Rabiger & Joullie 1964) Many benzimidazole derivatives give rise to two absorption bands in the range 220—360 nm. One band occurs at 240—250 nm and the other, more

present at the dose given Because of the quantitative treatment of the material all cases displaying vomiting have been sorted out

Little is known about the pharmacologic action of FB Certain data concerning the toxicity in rats have been given by Kimmerle (unpublished results) indicating an LD_{50} of 1100 mg per kg body weight, following oral administration In prolonged tests no increase in weight of the liver, kidneys, adrenal, and thyroid glands could be observed at a dosage of 60 mg per kg body weight, given 5 days a week over 2 months

The symptoms originating from the alimentary tract as observed in dogs in this investigation have also been reported in dogs receiving TBZ in oral doses larger than 200 mg per kg body weight (Robinson et al 1965) In these cases as well the vomiting coincided with the peak concentration of the drug in the blood The effects were believed to be attributable to disturbances of the central nervous system There are hence reasons to believe that the vomiting in dogs given FB is caused by the action of the compound on the central nervous system

METABOLITE DETECTION BY UV ABSORPTION

A comparison of the UV curves (280 nm) pertaining to column chromatographic fractionations of urine samples from animals before and after administration of FB shows in the latter case UV absorbing fractions that have no counterpart in the former (cf Figs 6—10) This would suggest that FB metabolites had been eluted in these UV absorbing fractions The presence of metabolites in the effluents was confirmed when the column eluates were examined by UV spectrophotometry and UV spectra of fractions with elution volumes of the same size (V_e) from the two column separations were compared This comparison indicated that the eluates from urine samples after FB administration contained fractions giving UV spectra that were completely absent in corresponding fractions (i e fractions with V_e of the same size) from urine samples from untreated animals Fractions pertaining to the same peak in the UV curve (280 nm) and possessing identical UV spectra were considered to contain the same metabolite they were consequently pooled Pooled fractions characterized by identical V_e values also possessed identical UV spectra and were hence considered to contain the same metabolite independent of their origin (i e, species) This assumption was corroborated by TLC examination of fractions of urine from a number of species as described above The examination showed that metabolites having identical V_e and identical UV spectra were also characterized by identical R_f values in various solvent systems (cf Table 7), independent of the organism in which the metabolites had been formed

Figs 12—19 show the UV spectra of the urinary metabolites observed in the present investigation The metabolites are designated by upper case letters in the same order as they were eluted from the column (cf Table 6) (The corresponding pooled fractions, which had the same V_e values as the metabolites were denoted similarly by P_A P_B, etc irrespective of the presence of metabolites)

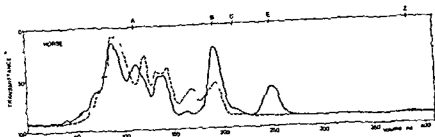


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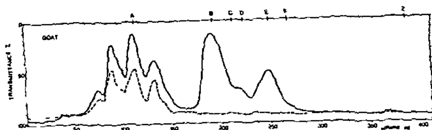


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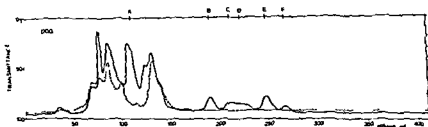


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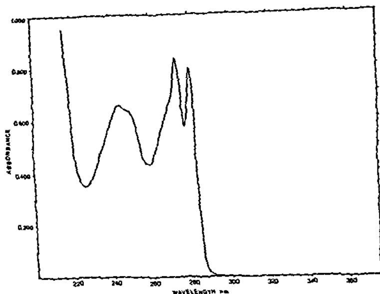


Fig 12 — UV spectrum of metabolite A in ammonium hydrogen carbonate buffer (0 1 M, pH 8) 25 μg/ml

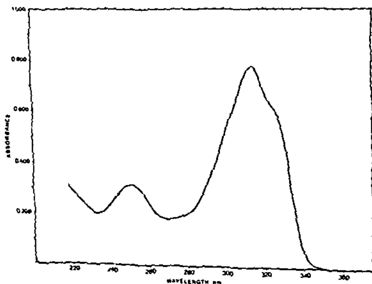


Fig 13 — UV spectrum of metabolite B in ammonium hydrogen carbonate buffer (0 1 M, pH 8) 10 μg ml

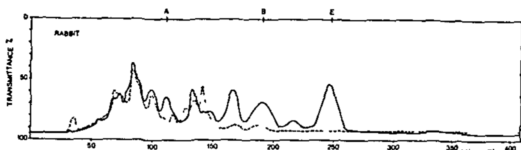


Fig 9 — Chromatographic elution pattern of one ml of rabbit urine (24 h) at an elution rate of 2 5 ml/h — After administration of a single oral dose of 97 5 mg FB per kg body weight - - - Normal urine (Otherwise as in Fig 6)

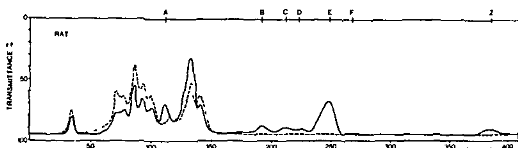


Fig 10 — Chromatographic elution pattern of two ml of rat urine (24 h) at an elution rate of 2 7 ml/h — *After administration of a single oral dose of 100 mg FB per kg body weight* Normal urine (Otherwise as in Fig 6)

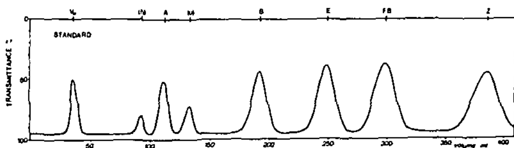


Fig 11 —Chromatographic elution pattern of an artificial test mixture in ammonium hydro gen carbonate buffer (0 1 M, pH 8, 1,5 ml) at a flow rate of 2 4 ml/h The absorption of the effluent was measured at 280 nm The composition of the mixture was as follows (amount of compound, mg, elution volume, ml)

blue dextran (2 0, 35 1=V_O, void volume)
hippuric acid (h) (1 8, 92)
metabolite A (1 1, 111)
uric acid (u) (0 1, 132)
metabolite B (1 1, 191)
metabolite E (1 1, 248)
FB (0 8, 298)
metabolite Z (=HOFB) (1 8, 385)

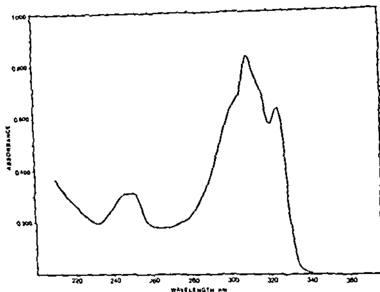


Fig 16 — UV spectrum of metabolite E in ammonium hydrogen carbonate buffer (0 1 M; pH 8) 8 µg/ml

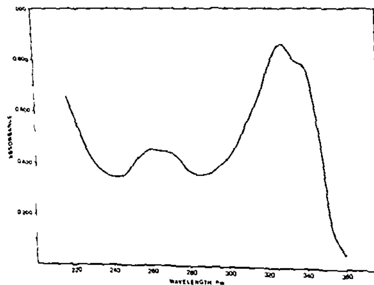


Fig 17 — UV spectrum of metabolite F_1 in ethanol (96 %)

3

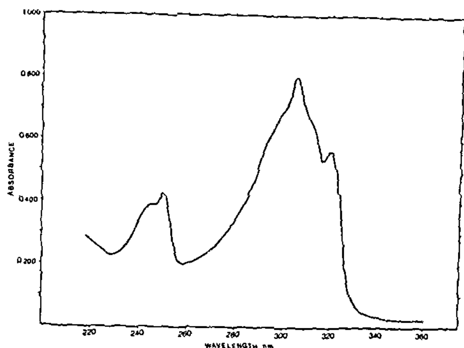


Fig 14 — UV spectrum of metabolite *C* in ammonium hydrogen carbonate buffer (0 1 M pH 8)

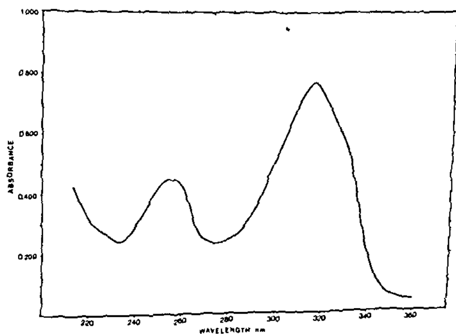


Fig 15 — UV spectrum of metabolite D in ammonium hydrogen carbonate buffer (0 1 M, pH 8)

intense band, at about 270—280 nm. The position and the fine structure of the absorption bands, especially that at longer wavelengths are influenced by substitution in the benzimidazole moiety (cf Leandri et al 1955, Rabiger & Joullie 1964)

CHARACTERIZATION OF METABOLITES BY R_f VALUES

Table 7 shows R_f values of the metabolites in various solvent systems and colour reactions It could be demonstrated by TLC that among the metabolite-containing eluates one fraction only, P_F, contained more than one metabolite, viz, metabolites F_1 and F_2 Since these metabolites are present only in very small quantities and the amount of F_2 may be neglected as compared to F_1, no difference was made between the two Metabolite F, hence, includes both F_1 and F_2 as occurring in the pooled fraction P_F

CHARACTERIZATION OF METABOLITES BY K_D VALUES

Table 6 shows the elution volumes (V_e) and distribution coefficients (K_D) of the metabolites, FB, hippuric acid and uric acid All K_D values are greater than 1 0, demonstrating that the compounds interact with or become adsorbed by the gel matrix

Metabolite Z (K_D=9 1) is eluted last from the column, whereas metabolite A (K_D=2 0) is the one first eluted from the column of all metabolites studied in this investigation As can be seen from Fig 11, and Table 6 the elution rate (K_D value) of metabolite A lies between the corresponding values of hippuric acid and uric acid These two acids separate in a similar manner in chromatographic separation of human urine on Sephadex G 10 in phosphate buffer (Sinha & Gabrieli 1968) elution with distilled water results in much lower resolution (Burtis et al 1970) For metabolites B C, D, E and F, K_D values between 4 and 6 were observed

The application of Sephadex dextran gel to adsorption chromatography of aromatic and heterocyclic compounds is well founded (Gelotte 1960, Beling 1961, Determann & Walter 1968 Brook & Housley 1969, Kowalska 1969, Khym & Uziel 1970) Dextran gels are inert and well suited to separation of delicate compounds of biological origin Starting from the assumption that at least part of the urinary FB metabolites would be aromatic (heterocyclic) compounds of unknown structure and chemical nature it was considered suitable to separate such compounds under mild conditions using Sephadex gel.

QUALITATIVE ASPECTS OF METABOLITE EXCRETION PATTERN IN DIFFERENT ANIMAL SPECIES

Applying the procedure described in the present chapter, i e, column chromatographic fractionation of urine and UV spectrophotometry of column eluate, several metabolites have been observed in urine from various animal species, following

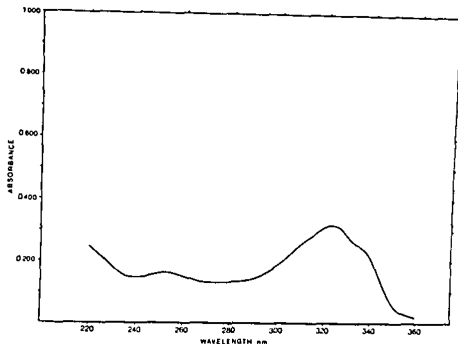


Fig 18 — UV spectrum of metabolite F_2 in ethanol (96 %)

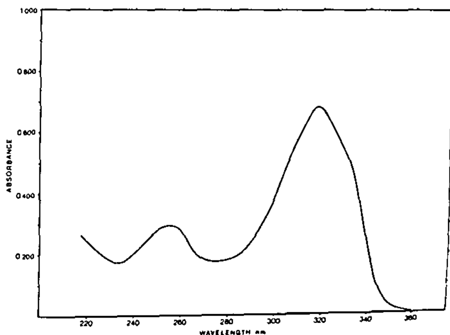


Fig 19 — UV spectrum of metabolite Z (=HOFB) in ammonium hydrogen carbonate buffer (0 1 M, pH 8), 5 μg/ml

intense band, at about 270—280 nm The position and the fine structure of the absorption bands, especially that at longer wavelengths, are influenced by substitution in the benzimidazole moiety (*cf* Leandri *et al* 1955, Rabiger & Joullié *1964)*

CHARACTERIZATION OF METABOLITES BY R_f VALUES

Table 7 shows R_f values of the metabolites in various solvent systems and colour reactions It could be demonstrated by TLC that among the metabolite-containing eluates one *fraction only*, P_F, contained more than one metabolite, *viz*, metabolites F_1 and F_2 Since these metabolites are present only in very small quantities and the amount of F_2 may be neglected as compared to F_1, no difference was made between the two Metabolite F, hence, includes both F_1 and F_2 as occurring in the pooled fraction P_F

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Table 6 shows the elution volumes (V_e) and distribution *coefficients* (K_D) of the metabolites, FB, hippuric acid and uric acid All K_D values are greater than 1 0, demonstrating that the compounds interact with or become adsorbed by the gel matrix

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The application of Sephadex dextran gel to adsorption chromatography of aromatic and *heterocyclic compounds is well founded* (Gelotte 1960 *Beling 1961*, Determann & Walter 1968, Brook & Housley 1969, Kowalska 1969, Khym & Uziel 1970) Dextran gels are *inert* and well suited to separation of delicate compounds of biological origin Starting from the assumption that *at least* part *of the* urinary FB metabolites would be aromatic (*heterocyclic*) compounds of unknown *structure* and chemical nature, it was considered suitable *to separate such* compounds under mild conditions using Sephadex *gel*

QUALITATIVE ASPECTS OF METABOLITE EXCRETION PATTERN IN DIFFERENT ANIMAL SPECIES

Applying the procedure described in the present *chapter*, *i e*, column chromatographic *fractionation of urine and UV* spectrophotometry *of column eluate*, several metabolites have been observed *in urine from* various animal species, following

administration of FB Depending on the species, the metabolite pattern in the urine varied both qualitatively and quantitatively Thus, urine from the goat and rat contained the greater number of metabolites (*cf* Figs 7 and 10), whereas urine from the dog was lacking in metabolite Z (*cf* Fig 8) In rabbit urine, only three metabolites (A, B and E) were detected (*cf* Fig 9), whereas horse urine contained not only A, B and E but also small amounts of metabolites C and Z (*cf* Fig 6) No unchanged FB (K_D=6 9) could be found in any of the urines examined The elution pattern of an artificial text mixture including FB as well is illustrated in Fig 11 No peak corresponding to FB can be seen in any of the Figs 6—10 which show the elution pattern of metabolite containing urine samples investigated

From a quantitative point of view only the relative occurrence of the metabolites could be noted In the urine of the animals tested in the course of this investigation metabolites A, B and E could be found in most cases in appreciable amounts while the other metabolites seemed to play only a minor role in a quantitative sense For this reason metabolites A B and E were considered to be the main metabolites of the degradation of FB in the mammalian organism

SUMMARY

A method of analysis has been developed for the purpose of separating and detecting FB metabolites in samples of urine The procedure comprises chromato graphy of the sample on a Sephadex column, continuous recording of the UV absorption of the eluates at 280 nm and subsequent UV spectrophotometric exam inations of the eluates The investigations were carried out on urine samples from experimental animals before and after FB administration A comparison of UV curves (280 nm) and UV spectra of eluates with identical elution volumes from the two separations disclosed the presence of FB metabolites in the urine following FB administration

It has proved possible by means of this procedure to demonstrate the presence of a number of metabolites in urine samples from the horse goat dog rabbit and rat after oral administration of FB The presence of common metabolites and the differences in the metabolite pattern of the urines from the various species are commented upon

Chapter 4. Comparative investigations of the urinary metabolite pattern in the horse, goat, dog, rabbit, and rat

Chapter 3 describes a method of separation and detection of urinary FB metabolites in various animal species, following oral administration of the compound. Certain variations in the occurrence of metabolites in the urine of the various species have been noted. Three of these metabolites, designated as metabolites A, B and E, were found in all urines independent of the species of the experimental animal. These metabolites as well as metabolite Z were consequently, subjected to isolation and structural analysis (Chapters 5–7, 8, and 9).

As can be seen from the latter part of the present work, the investigations proved metabolite Z to be a phenol (HOFB) that is formed in the enzymatic hydroxylation of the benzene nucleus of FB and metabolites B and E to be glucuronic acid and sulphuric acid conjugates of this phenol, respectively. Thus metabolite Z is 2-(2-furyl)-5(6)-hydroxybenzimidazole (HOFB) (Chapter 5), metabolite B is the β-glucuronide of HOFB (Chapter 7), metabolite E is a sulphate ester of HOFB (Chapter 8), and metabolite A is (S)-(—)-4-(2-benzimidazolyl)-4-hydroxybutyric acid (Chapter 9), resulting from the oxidative cleavage of the furan nucleus of FB.

The present section contains a description of quantitative investigations of these metabolites in the urine by means of their UV characteristics.

EXPERIMENTAL

The investigations were carried out on urine samples obtained during the animal experiments detailed in Chapter 3 (*cf.* Table 5, p. 24).

The UV absorption data, such as extinction coefficients and wavelengths of absorption maxima (λ_{max}) of the various metabolites, will be described in connection with the structural investigations of the metabolites (Chapters 5–7, 8, and 9).

EVALUATION OF METABOLITES. CORRECTIONS FOR INTERFERING ABSORPTION

General comments. — In the chromatographic separation of the urinary metabolites the eluates were divided by means of a fraction collector into equally large fractions of known volume, which were examined individually on a recording spectrophotometer (Beckman Model DK 2) in the 220—360 nm range. The concentration of the identified metabolites was evaluated at their maximum absorption, using the corresponding extinction coefficients. The shape of the UV absorption curve disclosed any irrelevant absorption and a corresponding correction was made (*direct determination*).

Table 8 — Determination of metabolite A in the *effluent* from column chromatographic separation of urine from the horse and dog after a single oral dose of FB (35 1 mg and 71 2 mg respectively per kg body weight) applying UV absorption spectrophotometry (1) directly on the eluate and (2) after TLC purification of the fractions

Species	Metabolite A % of dose	
	direct (1)	TLC (2)
horse	16 4	18 2
dog	22 3	22 9

Where strong interference of the UV absorption occurred from substances naturally present, TLC was used to purify the metabolite After its elution with ethanol from the adsorbent the quantitative analysis followed using UV spectro photometry *(determination after TLC)*

Metabolite A — In the evaluation of metabolite A a correction had to be made at the direct determination in the column eluate The absorption at $\lambda_{max}=280$ nm was used for quantitative determination As can be seen from Fig 12 which shows the UV spectrum of metabolite A there is a steep drop in the absorption above 280 nm with no absorption at all at and above 295 nm This change in UV absorption could be used successfully in the correction for interfering absorption

In column chromatography of urine samples from untreated animals fractions of the same elution volume as metabolite A commonly show UV spectra with broad absorption maxima at about 280 nm There is a slow decrease in the ab sorption as the wavelength increases but the absorption is still noticeable at 320 nm Since metabolite A does not absorb above 295 nm the course taken by the absorp tion curve at this wavelength provides information concerning the absorption from contaminants that may occur Knowing the shape of the UV curve of the contaminants a correction can be made by extrapolation of the curve from longer wavelengths to 280 nm

In the case of horse and dog urine the quantitative determinations were carried out both directly on the column eluates applying the appropriate correction and after TLC separation with solvent I (Table 7 p 27) A comparison of the analytical results (Table 8) shows satisfactory agreement between the values obtained by direct determination on column eluates and those obtained following TLC treatment Direct determinations could be made for all urines except the samples of goat urine in which naturally occurring products resulted in strong interference with metabolite A making it necessary to introduce TLC separation prior to the spectrophotometric determination

Metabolites B E and Z — The evaluation of metabolites B E and Z was not disturbed by UV absorption caused by naturally occurring substances in the urine The reason is that fractions containing these metabolites seldom contain contami nants as well and where they do occur they do not affect the evaluation

In horse urine UV absorbing substances are eluted together with metabolite B (Fig 6) In these fractions the UV absorption of the contaminants has its maximum at 280 nm The absorption decreases rapidly at longer wavelengths and no absorption remains at 300 nm Since the reading of the UV absorption in the evaluation of metabolite B is carried out at $\lambda_{max}=314$ nm (*cf* Fig 13), the presence of these contaminants will not interfere with the quantitative determination The evaluation of metabolites E and Z is performed at $\lambda_{max}=309$ and 318 nm respectively (*cf* Figs 16 and 19) The irrelevant absorption influencing the determinations was insignificant.

Metabolites C D and F — The amount of metabolites C, D and F, *i e*, unidentified metabolites, was estimated by applying the mean values of the extinction coefficients for metabolites B, E and Z and reading at $\lambda_{max}=305$ nm for C, 315 nm for D and 327 nm for F (*cf* Figs 14, 15 and 17) This method, of course, gives the values a *relative* character only and excludes their being used in a strictly quantitative sense However, they can be used as an indication of the presence and formation of these metabolites, the extent of which varies depending on the animal species

RESULTS

Six *dogs* were given FB orally in various amounts (65—169 mg per kg body weight) The urine of all of these animals was collected for 24 h Four of the dogs were retained in metabolism cages for an additional 24 h The results are summarized in Table 9

In the course of the first 24 h five out of six dogs excreted 28 per cent (25—30 per cent) of the administered dose in the form of metabolites A, B and E

The urine collected from four dogs during 48 h, which contained 32 per cent (31—34 per cent) of the dose, is represented diagrammatically in Fig 20 In the dog the main metabolite was A The sum total of metabolites B and E varied between 0 7 and 2 0 per cent of the dose given The overall quantity of the other metabolites (C, D and F) was estimated to be close to the sum total of metabolites B and E As can be seen in Table 9 the percentage amount of the FB dose that could be detected in metabolized form was constant as was the ratio of metabolite A to the other metabolites at the various dosages of FB applied in these experiments

The *horse goat* and *rabbit* species are represented by one animal each whereas *in the experiments on rats* three animals were used for collecting pooled urine In these species as well the main part of the metabolites was excreted during the first 24 h (*cf* Table 9) The overall amount of metabolites A, B, E and Z in urine collected during 48 h as expressed as a percentage of the dose administered was 54 per cent in the rabbit, 40 per cent in the horse (18 h), 32 per cent in the goat and 21 per cent in the rat The results have been compiled in Table 9 and Fig 20 An important part of the degradation products even in these species, metabolite A did not dominate the metabolite pattern to the same extent as in the

Table 9 — Metabolites of FB in urine of various species The values given for each metabolite are percentages of the dose excreted in 24 h Total= amount of metabolites excreted in 48 h +=trace, just detectable in some individuals Each test relates to urine collection from a single individual, except in the rat where urine was pooled from 3 animals Values in brackets are estimated

Species	Dose mg/kg	Urine collection days after dosing	% of dose recovered in the urine after administration of FB									
			Metabolites							Metabolites, total		
			A	B	C	D	E	F	Z	A+B+E+Z	B+E+Z	C+D+F
horse	35 1	after 18 h	18 2	16 8	(0 2)	—	4 3	—	0 2	39 5	21 3	(0 2)
goat	82 4	1st	10 4	12 9	(0 4)	(0 8)	3 7	(0 3)	0 3	27 3	16 9	(1 5)
		2nd	2 4	1 2	(+)	(0 5)	0 4	(0 1)	0 6	4 6	2 2	(0 6)
		Total	12 8	14 1	(0 4)	(1 3)	4 1	(0 4)	0 9	31 9	19 1	(2 1)
rabbit	97 5	1st	18 7	12 7	—	—	22 7	—	—	54 1	35 4	—
		2nd	—	+	—	—	+	—	—			
		Total	18 7	12 7	—	—	22 7	—	—	54 1	35 1	—
rats	100 0	1st	10 2	1 0	(0 7)	(0 7)	7 2	(0 4)	1 5	19 9	9 7	(1 8)
		2nd	0 6	0 1	—	(+)	0 1	—	+	0 8		
		Total	10 8	1 1	(0 7)	(0 7)	7 3	(0 4)	1 5	20 7	9 7	(1 8)

										A+B+E	B+L	C+D+F
dog	100 0	1st	28 1	0 6	(0 2)	(0 5)	1 0	(0 5)	—	29 7	1 6	(1 2)
mongrel beagle	71 2	1st	22 9	0 9	(0 4)	(0 5)	1 0	(0 2)	—	24 8	1 9	(1 1)
beagle	65 2	1st	9 1	0 1	(0 1)	(0 2)	0 1	(+)	—	9 3	0 2	(0 3)
		2nd	23 6	0 3	(0 4)	(0 6)	0 3	(0 3)	—	24 2	0 6	(1 3)
		Total	32 7	0 4	(0 5)	(0 8)	0 4	(0 3)	—	33 5	0 8	(1 6)
beagle	80 1	1st	28 0	0 2	(0 4)	(0 6)	0 4	(0 5)	—	28 6	0 6	(1 5)
		2nd	1 8	+	(0 1)	(0 5)	0 1	(0 2)	—	1 9	0 1	(0 8)
		Total	29 8	0 2	(0 5)	(1 1)	0 5	(0 7)	—	30 5	0 7	(2 3)
beagle	91 8	1st	27 3	0 4	(0 4)	(0 4)	0 8	(0 2)	—	28 5	1 2	(1 0)
		2nd	3 5	0 1	(0 1)	(0 4)	0 1	(0 2)	—	3 7	0 2	(0 7)
		Total	30 8	0 5	(0 5)	(0 8)	0 9	(0 4)	—	32 2	1 4	(1 7)
beagle	168 6	1st	27 0	0 8	(0 3)	(0 2)	1 0	(0 3)	—	28 8	1 8	(0 8)
		2nd	2 7	0 1	(+)	(0 1)	0 1	(0 2)	—	2 9	0 2	(0 3)
		Total	29 7	0 9	(0 3)	(0 3)	1 1	(0 5)	—	31 7	2 0	(1 1)

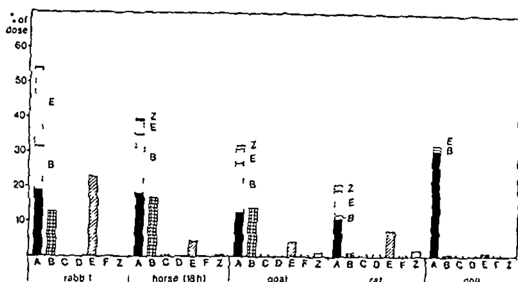


Fig 20 — Urinary excretion of FB metabolites during 48 h in various species expressed as per cent of a single oral dose The metabolites A, B, E, and Z were determined using known extinction coefficients, the unidentified metabolites C, D and F were only estimated (see text) The cumulative excretion as well as the individual metabolites are indicated The values for the dog are the mean of four animals

dog In these species, considerable quantities of HOFB were excreted, mostly in conjugated form (metabolites B and E) but to a certain extent in free form as well (metabolite Z in the rat, goat and horse) The metabolite pattern in the various species examined and the ratio of metabolite A to the other metabolites are illustrated in Fig 20 The ratio between metabolite A and the sum total of metabolites B, E and Z varies in the different animal species as follows (per cent of the dose) in the horse, 18/21, goat, 13/19, rabbit, 19/35, rat, 11/10 The other metabolites (C, D and F) are of less importance from a quantitative point of view, and this applies to the dog as well

DISCUSSION

The present investigation reveals considerable differences in the urinary metabolite pattern among the various species used

Following oral administration of various dosages of FB dogs reveal conditions of a remarkable constancy with respect to the metabolite pattern and the percentages of the various metabolites in the urine (*cf* Table 9) The main metabolite is A, which is formed by the cleavage of the furan ring This metabolite amounts to 31 per cent of the FB dose and is the highest percentage that has been determined in any of the species investigated The metabolism also occurs by hydroxylation of the benzene nucleus as can be concluded from the presence of metabolites B and E, which are conjugates of HOFB The overall amount of B and E in the dog is the lowest found in the five species (*cf* Fig 20) The metabolite pattern in the dog

shows a broad spectrum. Other compounds also, such as metabolites C, D and F, which have not yet been identified, are formed in small quantities Metabolite Z, however, which is unconjugated HOFB, was not observed in the dog on any occasion

In the *rabbit* the metabolite pattern is comparatively simple HOFB is the main metabolite and is excreted only in the conjugated form as metabolites B and E Together, these two metabolites alone amount to one third of the dose In the rabbit experiment, more of the sulphate ester (metabolite E) than of the glucuronide (metabolite B) of HOFB was excreted Metabolite A constituted one-third of the total amount of metabolites No other metabolites were observed

While the metabolite pattern in the rabbit is comparatively simple, *rats* show a more complicated pattern, including a number of metabolites (C, D and F) that have not yet been identified The overall amount of metabolites A, B, E and Z was found to be only 21 per cent of the dose given, one half of the total being metabolite A and the other half compounds originating from HOFB Most of the excreted HOFB was conjugated in the form of the sulphate ester (metabolite E) but it also occurred in the unconjugated state (3 per cent of the dose)

The excretion in the *goat,* which is a ruminant, seems to be slower than in the other animal species investigated here This view seems to be corroborated by the fact that relatively large quantities of metabolites were excreted even during the second 24 h period The overall quantity of metabolites in the goat amounts to one third of the dose less than one half of this quantity being metabolite A and more than one half being made up of conjugated (metabolites B and E) and free HOFB The greater part of the conjugates is glucuronide (metabolite B) Of the five species of experimental animals in the present investigation, the goat and the rat possess the most diversified metabolite pattern

A comparison of the FB metabolite pattern in the *horse* and goat shows similarities in the excretion as far as the ratio between metabolite A and the total amount of metabolites B and E is concerned Thus, slightly more than one half of the metabolites consists of HOFB conjugates In both species the main part is metabolite B (glucuronide) Among the other metabolites (C, D, F, and Z) excreted with the goat urine, metabolites C and Z were found to be present in the horse urine in minute quantities only The sum total of the metabolites found in the horse during 18 h is greater than that observed in the goat during 48 h, indicating faster excretion in the former species

The *small amounts of metabolites B and E in the dog* may be explained by the oxidative metabolism of the benzene nucleus proceeding beyond the formation of HOFB It is also suggested as an alternative explanation that the hypothetic intermediates (arene oxides and dihydrodiols 1, 2, 3, 4 in Diagram 2 on p 55), may be further metabolized along another route than the one leading to HOFB in the diagram. In both cases a continuation of the metabolic process may lead to a cleavage of the benzene nucleus Metabolic splitting of the benzene nucleus has been discussed by Williams (1959 *a*) and others The view just mentioned is

supported to some extent by the fact that another benzimidazole derivative as well, TBZ, is metabolized differently in the dog than in the other species (Tocco *et al* 1966) In an investigation of the metabolism of TBZ in rats, using a compound that was labelled with ^{14}C in the benzene nucleus, Rosenblum *et al* (1964) noticed the presence of $^{14}CO_2$ in the expiration air of rats This observation corroborates the theory of the progressive degradation of the benzene nucleus Possibly, this de gradation may occur on a larger scale in the dog than in the other species, which would explain the species difference between the TBZ metabolite patterns It would seem reasonable to assume that the metabolism of FB follows the same lines

In the dog, only small amounts of metabolites B and E are excreted In the other species examined, on the contrary, the excreted amounts of these metabolites are considerable *In the course* of this investigation it was further demonstrated that in the species examined variations were noted not only in the total amount of metab olites B and E (and Z) but also in the ratio between metabolites B and E It is well known, however, that the conjugation of phenols with glucuronic acid and sulphuric acid is affected by a number of factors, such as the dosage level, diet and temperature in the experiments performed, (*cf* Williams 1959, *b*), which explains why the ratio between the glucuronic acid and sulphuric acid conjugate will vary even in the individual animal according to the experimental conditions

The *ratio* between metabolites B and E, consequently, is less important in evaluating the experimental results than the sum total of these metabolites, which, together with the quantity of free phenol (HOFB), indicates the part of the FB dose that has been metabolized and excreted as HOFB and/or its conjugates

Small amounts of *metabolite* Z (HOFB) were found in the horse, goat and rat urine No *unchanged FB* could, however, be observed in any of the urines in vestigated, as mentioned in Chapter 3 on p 36 When these compounds (FB, HOFB) are absent in the urine, this may be explained by the fact that the lipophilic character of a chemical compound determines both the absorption by gastro intestinal organs and the renal excretion Owing to the lipophilic properties of FB and HOFB, these substances are easily reabsorbed in the tubules, following glomerular filtration

It has not been possible to decide with certainty whether the unconjugated HOFB in the horse, goat and rat is excreted through the kidneys or if it is formed secondarily in the urine by hydrolysis of an excreted conjugate Investigations (Conzelman *et al* 1970) have shown that there may be a risk of misjudgement in metabolite studies, owing to the natural occurrence of sulphatase and β glu curonidase in the urine The experimental conditions of the present work make it impossible to completely rule out any such effect or spontaneous hydrolysis (or any microbial activity for that matter) since the urine samples were kept at the ambient temperature during the period of collection It does seem however as if the occurrence of HOFB in goat and rat urine is of such an order of magnitude as to suggest excretion by the kidneys In studies of TBZ metabolism (Tocco *et al* 1965, 1966, McManus *et al* 1966), various quantities both of the initial com

pound and of the unconjugated 5(6) hydroxy 2 (4-thiazolyl)benzimidazole were observed in urine samples from a number of animal species The excreted amounts of this phenol were considerably greater than the amounts of HOFB in the present investigation

SUMMARY

The data (UV spectra) obtained from the qualitative investigation of horse, goat, dog, rabbit, and rat urine have been utilized in a quantitative evaluation by means of extinction coefficients of the individual metabolites Although somewhat time-consuming, the procedure was nevertheless quite useful as a means of evaluating the urinary metabolites The experimental results were used in a comparative study of the metabolic pattern of different species

The investigation confirmed that the main urinary metabolites of FB in all species examined were metabolites A, B and E Metabolites B and E are both conjugates of the phenolic metabolite Z (HOFB) Other metabolites detected were quantitatively unimportant

Only small amounts of unconjugated metabolite Z were found in horse, goat and rat urine

In the rabbit, 54 per cent of the dose was excreted in 48 h, while in the other species the amounts excreted during the same period were less, *viz*, in the horse (18 h), 40 per cent, goat, 32 per cent, dog, 32 per cent, and rat, 21 per cent. The dose of FB administered had no effect on the percentage of metabolites excreted in the dog

There are certain noticeable differences in the excretion pattern of the dog compared with those of the other species, especially as concerns the occurrence in dog urine of large quantities of metabolite A and of only very small quantities of metabolites B and E In fact more than 90 per cent of the total metabolite excretion in the dog was in the form of metabolite A In all other species investigated, the conjugates of the phenol, HOFB, *i e*, metabolites B and E, had a larger share in the overall amount of excreted metabolites although here as well the amount of metabolite A was significant. The ratio between metabolite A and the HOFB conjugates was thus found to range between 1 1 in the rat and 1 2 in the rabbit The corresponding values were intermediate in the horse and goat

Chapter 5. Isolation and identification of metabolite Z

Samples of urine from goat were chromatographed on a Sephadex column prior to and following administration of FB (*Chapter 3*) A comparison of the UV curves for the column effluents (Fig 7) showed that only urine collected after administration of FB contained a UV absorbing fraction (P_z), which was slowly eluted from the column (K_D=9 1, *cf* Table 6, p 26) There was a certain similarity between the UV spectrum of this fraction (Fig 19) and that of FB (Fig 2) suggesting that the fraction contained a metabolite, denoted here as metabolite Z (Chapter 3) The present chapter contains a description of the isolation and identification of this metabolite

EXPERIMENTAL AND RESULTS

APPARATUS

The UV spectra were obtained on a Beckman Model DK 2 recording spectrophotometer, using 10 mm quartz cells Solvents to determine values for calculation of log ε were chosen in accordance with those used for standardized UV measurements of benzimidazole compounds (Rabiger & Joullie 1964)[8] The log ε data are uncorrected

The IR absorption spectra were recorded on a Perkin Elmer Model 337 Grating Infrared spectrophotometer All compounds were studied in the solid state in pressed disks of potassium bromide

The mass spectra were measured on an LKB 9000 mass spectrometer using the direct inlet system The ion source temperature was about 270° and the energy of the ion beam was 70 eV The data were plotted by an IBM 1800 computer

The NMR spectra were obtained on a Perkin Elmer Model R 12 (60 MHz) and a Varian HA 100 (100 MHz) spectrometer in micro cells using hexadeuterodimethylsulphoxide (DMSO d_6) as solvent and tetramethylsilane (TMS) as internal standard unless otherwise stated All values are δ (ppm) relative to TMS=0

The melting points were determined on a melting point microscope with a Kofler micro hot stage (C. Reichert Vienna Austria) The values are uncorrected

[8] 1×10^{-4} M solutions were usually prepared with absolute ethanol hydrochloric acid (0 01 M pH 2 0) and sodium hydroxide (0 01 M pH 12 1) As a neutral solution a phosphate buffer (pH 7 0 μ=0 1) was used with the same concentration of the test compound as indicated above

The elementary analyses were carried out, applying ultramicroanalytical techniques, in the Microanalytical Laboratory of the Royal Agricultural College, Uppsala, Sweden

MATERIAL

Goat urine was used for separation and isolation of metabolite Z according to the description of column chromatography in Chapter 3 (p 25)

ISOLATION AND STRUCTURAL INVESTIGATION

A sample of goat urine (2 ml) collected during the second 24 h period after the administration (*cf* Table 5, p 24) was chromatographed on a Sephadex column (*cf* Chapter 3, p 25) Following the passage of elution buffer (362 ml) through the column, fractions were eluted which exhibited absorption maxima at wavelengths 318 and 255 nm (Fig 19) Fractions showing such UV spectra were combined and the pooled fractions (44 ml) were extracted with equal volumes of ethyl acetate (3 times) The combined extracts were dried over anhydrous sodium sulphate and evaporated in a rotary evaporator at reduced pressure and at ambient temperature

The residue after evaporation was dissolved in methanol (300 μl) The solution (2×5 μl) was chromatographed on two thin layer plates, using solvent systems II and III, respectively (*cf* Table 7, p 27) Blue spots appeared on examination in UV light (254 nm) The R_f values of the two spots were 0 75 and 0 40 in the respective solvents (*cf* Table 7)

The remaining portion of the solution was band chromatographed on two thin-layer plates, using solvent system II Following drying in the air, the band corresponding to $R_f = 0\ 75$ was scraped off The substance was eluted with ethanol and concentrated to dryness under nitrogen The residue was dissolved in methanol (100 μl), evaporated on potassium bromide and examined by micro IR spectroscopy The IR spectrum was found to be almost identical with that of synthetic HOFB (*cf* Fig 22) After IR spectroscopy the potassium bromide disk, containing metabolite Z, was dissolved in a few drops of water, the turbid aqueous solution becoming clear on addition of methanol (1 ml) For UV measurements equal volumes (150 μl) of this solution were transferred to quartz cells and diluted with (1) hydrochloric acid (0 1 M), (2) sodium hydroxide (0 1 M) and (3) ammonium hydrogen carbonate buffer (0 1 M) The total volume of the test solution was in each case 2 ml The spectra of metabolite Z in these solutions had absorption maxima at wavelengths 328 340 and 317 5 nm, respectively The absorption maximum for metabolite Z in ethanol was 321 nm (*cf* Table 10)

SYNTHESIS OF 2 (2 FURYL) 5(6) HYDROXYBENZIMIDAZOLE (HOFB)

In analogy with the synthesis of 5 hydroxythiabendazole (Tocco *et al* 1964), 2 furoyl chloride was reacted with 5 methoxy 2 nitroaniline to form the corre-

Chapter 5. Isolation and identification of metabolite Z

Samples of urine from goat were chromatographed on a Sephadex column prior to and following administration of FB (Chapter 3). A comparison of the UV curves for the column effluents (Fig. 7) showed that only urine collected after administration of FB contained a UV absorbing fraction (P_z), which was slowly eluted from the column (K_D=9.1, *cf.* Table 6, p. 26). There was a certain similarity between the UV spectrum of this fraction (Fig. 19) and that of FB (Fig. 2), suggesting that the fraction contained a metabolite, denoted here as metabolite Z (Chapter 3). The present chapter contains a description of the isolation and identification of this metabolite.

EXPERIMENTAL AND RESULTS

APPARATUS

The UV spectra were obtained on a Beckman Model DK 2 recording spectrophotometer, using 10 mm quartz cells. Solvents to determine values for calculation of log ε were chosen in accordance with those used for standardized UV measurements of benzimidazole compounds (Rabiger & Joullie 1964)[8]. The log ε data are uncorrected.

The IR absorption spectra were recorded on a Perkin Elmer Model 337 Grating Infrared spectrophotometer. All compounds were studied in the solid state in pressed disks of potassium bromide.

The mass spectra were measured on an LKB 9000 mass spectrometer using the direct inlet system. The ion source temperature was about 270° and the energy of the ion beam was 70 eV. The data were plotted by an IBM 1800 computer.

The NMR spectra were obtained on a Perkin Elmer Model R 12 (60 MHz) and a Varian HA 100 (100 MHz) spectrometer in micro cells using hexadeuterodimethylsulphoxide (DMSO d_6) as solvent and tetramethylsilane (TMS) as internal standard unless otherwise stated. All values are δ (ppm) relative to TMS=0.

The melting points were determined on a melting point microscope with a Kofler micro hot stage (C. Reichert, Vienna, Austria). The values are uncorrected.

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[8] 1×10^{-4} M solutions were usually prepared with absolute ethanol, hydrochloric acid (0.01 M, pH 2.0) and sodium hydroxide (0.01 M, pH 12.1). As a neutral solution a phosphate buffer (pH 7.0, μ=0.1) was used with the same concentration of the test compound as indicated above.

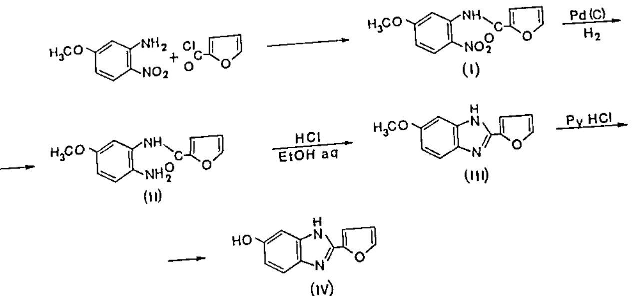


Diagram 1 — Synthesis of 2 (2 furyl) 5(6) hydroxybenzimidazole

sponding 2 nitrophenyl amide (I) (The scheme of synthesis is illustrated in Diagram 1) By catalytic hydrogenation with palladium on carbon the 2 nitrophenyl amide (I) was converted into 2 aminophenyl amide (II) The cyclization of this compound (II) to 2 (2 furyl) 5(6) methoxybenzimidazole (III) was effected by boiling with alcoholic hydrochloric acid Demethylation of this methyl ether to 2 (2 furyl) 5(6) hydroxybenzimidazole (IV) was accomplished by means of pyridine hydrochloride

N-(5-methoxy-2 nitrophenyl)-2-furancarboxamide (I)

2 furoyl chloride (2 8 g) was added to a solution of 5 methoxy 2 nitroaniline (3 4 g) in anhydrous pyridine (70 ml) in a flask fitted with a condenser carrying a calcium chloride tube at the top The mixture was heated (6 h) at 70° and then poured into water (600 ml) After 8 h the solid was collected by filtration and washed with dilute hydrochloric acid sodium hydrogen carbonate solution and finally with water The product was dissolved in hot ethanol (80 ml) and the solution treated with charcoal Long yellow needles separ cooling The yield was 4 5 g (86 per cent) m p 137 5—138° (N 10 7

$C_{12}H_{10}N_2O_5$ requires C 55 0, H 3 8

UV spectrum ethanol (abs)

IR spectrum (KBr) 3300 cm

N-(5 methoxy-2 aminophen

Compound (I) (5 2 g) abs ethanol (200 ml) atmospheric pressure most of it during the and the solution evaj was dissolved in hot (68 per cent), m p requires C 62 1, H

UV spectrum etl

IR spectrum (KI NH)

2-(2 furyl) 5(6)-m

Compound (II water (10 ml) a the crystals wer with acetone Tl recrystallization crystals became H 1 9, N 10 2 (

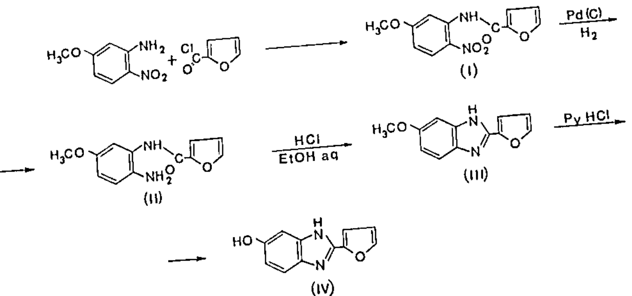


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UV spectrum ethanol (abs) λ_{max}=389, 267 nm, log ε=3 61 4 34

IR spectrum (KBr) 3300 cm^{-1} (amide NH), 1685 cm^{-1} (amide CO)

N-(5 methoxy-2-aminophenyl)-2-furancarboxamide (II)

Compound (I) (5 2 g) and 5 per cent palladium on carbon catalyst (1 g) in abs ethanol (200 ml) were shaken under hydrogen at ambient temperature and atmospheric pressure Hydrogen (1580 ml) was absorbed rapidly within 45 min most of it during the first 15 min The catalyst was then removed by centrifugation and the solution evaporated to dryness at 30° and reduced pressure The residue was dissolved in hot benzene Colourless plates separated on cooling yield 3 1 g (68 per cent) m p 146—148° (Found C 62 1 H 5 3, N 12 1 $C_{12}H_{12}N_2O_3$ requires C 62 1, H 5 2, N 12 1)

UV spectrum ethanol (abs) λ_{max}=297 241 nm log ε=3 84 4 25

IR spectrum (KBr) 3400 cm^{-1} (amine NH) 3318 cm^{-1} (amine NH and amide NH)

2-(2-furyl)-5(6)-methoxybenzimidazole hydrochloride monohydrate (IIIa)

Compound (II) (1 1 g) was refluxed (5 h) in a mixture of ethanol (20 ml) water (10 ml) and concentrated hydrochloric acid (4 ml) After 10—12 h at 0° the crystals were collected and washed with hydrochloric acid (1 M) and finally with acetone The yield was 1 3 g (83 per cent) Colourless plates separated after recrystallization from hydrochloric acid (1 M) Melting behaviour The transparent crystals became opaque above 100° and melted at 235—238° (Found C 53 5 H 4 9, N 10 2 $C_{12}H_{10}N_2O_2$ HCl H_2O requires C 53 6, H 4 9 N 10 4)

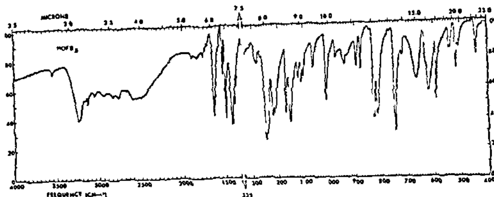


Fig 22 — IR spectrum of synthetic 2 (2 furyl) 5(6) hydroxybenzimidazole (*HOFB*) in potassium bromide disk.

g (78 per cent) melted between 135° and 150° with decomposition (Found C 48 5, H 4 7, N 10 0 $C_{11}H_8N_2O_2$ *HCl* $2H_2O$ *requires C 48 5, H 4 8, N 10 3*)

2 (2-furyl)-5(6) hydroxybenzimidazole (IVb) (HOFB)

Compound (IVa) (150 mg) was dissolved in water (3 ml) and ammonium *hydrogen carbonate solution* (1 M, 1 ml) was added The yellow precipitate was collected by filtration and washed with water (Quantitative yield) The yellow product melted at 264—266° after recrystallization from acetonitrile (Found C 66 0, H 4 2, N 14 0 $C_{11}H_8N_2O_2$ requires C 66 0, H 4 0, N 14 0)

The R_f values (TLC) characterizing HOFB (IV) in various solvent systems are shown in Table 7 on p 27 The K_D value of the compound was identical with that of metabolite Z (Table 6, p 26)

For UV spectra see Fig 21 and Table 10

The IR spectrum (KBr) is shown in Fig 22 The absorption band at 3540 cm 1 is caused by stretching vibrations of the phenolic OH group, absorption bands in the 3400—2100 cm 1 range are attributable to stretching *vibrations of inter and/or*

Table 10 — UV spectra of synthetic 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB) in various solvents and at different pH values

Solvent	λ_{max} nm (log ε)					
ethanol abs	321	(4 42)	256	(4 07)		
hydrochloric acid (0 01 M)	328	(4 45)	261 5	(4 06)		
sodium hydroxide (0 01 M)	340	(4 33)	263	(4 08)	234	(4 08)
phosphate buffer (μ=0 1 pH 7 0)	318	(4 43)	255	(4 07)		
ammonium hydrogen carbonate buffer (0 1 M pH 8 1)	318	(4 43)	255	(4 07)		

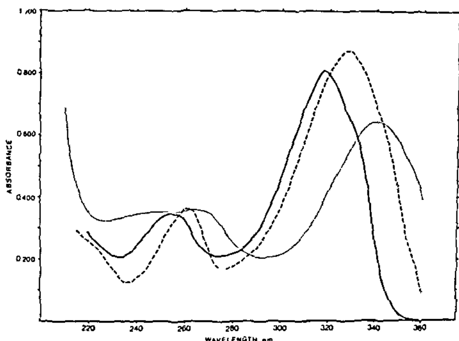


Fig 21 — UV spectra of synthetic 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB) in aqueous solutions at different pH values —— phosphate buffer (μ=0 1, pH 7 0), 6 μg/ml, hydrochloric acid (0 01 M), 6 5 μg/ml, sodium hydroxide (0 01 M), 6 0 μg/ml

2-(2-furyl)-5(6)-methoxybenzimidazole (IIIb)

Compound (IIIa) (2 0 g) was dissolved in water (100 ml), the solution was then chilled with ice and neutralized by the dropwise addition of a solution (10 ml) of sodium hydrogen carbonate (0 8 g) White crystals precipitated after 10—12 h at 0° and were collected by filtration and washed with water, yield 1 5 g (97 per cent) The colourless product melted at 152—154°, after recrystallization from cyclohexane (Found C 67 3, H 4 7, N 13 0 $C_{12}H_{10}N_2O_2$ requires C 67 3, H 4 7, N 13 1)

UV spectrum, ethanol (abs) λ_{max}=319, 256 nm, log ε=4 45, 4 05

IR spectrum (KBr) intense band at 747 cm 1, which is characteristic of 2 substituted benzimidazoles (cf Rabiger & Joullie 1964)

2-(2-furyl)-5(6)-hydroxybenzimidazole hydrochloride dihydrate (IVa)

Compound (IIIb) (1 5 g) was heated (45 min) with anhydrous pyridine hydrochloride (16 g) at 220—230° The mixture was dissolved in a small amount of water The precipitate, a straw coloured product, was recrystallized from hydro chloric acid (1 M) (charcoal) The yellow needles thus obtained in a yield of 1 5

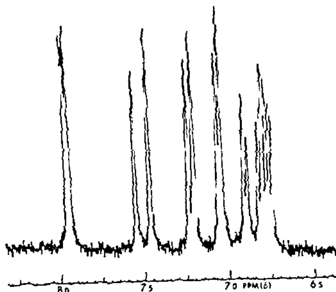


Fig 24 — NMR spectrum (100 MHz) of synthetic 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB) in d methylsulphoxide

The coupling constants observed for the three protons of the furan ring were in the range found for a series of 2 substituted furan derivatives investigated by Gronowitz *et al* (1963) The three remaining protons show the spin—spin coupling pattern of a 1 2 4 trisubstituted benzenoid fragment (Mathieson 1965)

DISCUSSION

A comparison of the R_f values of the metabolites of FB (*cf* Table 7 on p 27) suggested that metabolite Z was the least polar among the metabolites investigated, implying that this metabolite would also be the FB metabolite easiest to extract, which also was found to be the case After isolation by means of TLC the compound was subjected to UV and IR spectrophotometric investigations which were of primary importance to the structural elucidation of this metabolite

It may be assumed that metabolite Z is formed by enzymatic action on the benzene and/or furan nucleus of the FB A sharp absorption band at 885 cm^{-1}, in the IR spectrum of metabolite Z indicates however, that the furan nucleus is intact. The UV spectrum of metabolite Z (Fig 19) possesses no fine structure and shows an absorption maximum at 321 nm in ethanol The corresponding maximum for FB (Fig 2 Table 15) is at 307 nm These facts would suggest a substitution in the benzene nucleus of FB by one (or more) electron donating groups (*cf* Leandri *et al* 1955 Rabiger & Joullie 1964) It may therefore be assumed that metabolite Z is a phenolic derivative of FB containing one or more hydroxyl groups

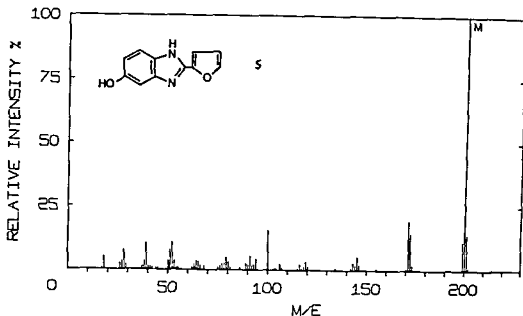


Fig 23 — Mass spectrum of synthetic 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB)

intramolecular hydrogen bonded OH and NH groups The absorption bands at 3120 cm 1 and 885 cm 1 have been attributed to the furan nucleus (Wrobel & Galuszko 1970)

The mass spectrum is illustrated in Fig 23 The base peak is the molecular peak M at *m/e* 200

NMR spectrum Compound (IVb) (70 mg) was dissolved in DMSO d_6 (0 4 ml) using TMS as an internal standard and the spectrum run at 60 MHz The spectrum displayed a broad singlet at $\delta = 9\ 5$ (2H, >NH and OH) and several signals in the region $\delta = 6\ 7$—$7\ 9$ Improved resolution of the spectrum was ob tained when run at 100 MHz (The compound was dissolved in DMSO and the solvent signal was used as internal lock) The spectrum is shown in Fig 24 The signal positions were measured with respect to the lock signal ($\delta = 2\ 62$)

Protons on the furan ring

(H_3) $\delta = 7\ 21$ doublet, $J_{3\ 4} - 3\ 4$ cps
(H_4) $\delta = 6\ 76$ quartet[9], $J_{3\ 4} = 3\ 4$ cps $J_{4\ 5} = 1\ 8$ cps
(H_5) $\delta = 7\ 94$ quartet,

Protons on the benzene ring

(H_4) $\delta = 7\ 04$ doublet $J_{4\ 6} = 2\ 0$ cps
(H_6) $\delta = 6\ 85$ quartet[9], $J_{4\ 6} - 2\ 0$ cps $J_{6\ 7} = 8\ 5$ cps
(H_7) $\delta = 7\ 50$, doublet, $J_{6\ 7} = 8\ 5$ cps

[9] The last upfield signal of the quartet of H_6 on the benzene ring coincided with the last downfield signal from the quartet of H_4 on the furan ring (*cf* Fig 24) In the 60 MHz spectrum one half of the quartet of H_6 coincided with the signal from H_4 on the furan ring

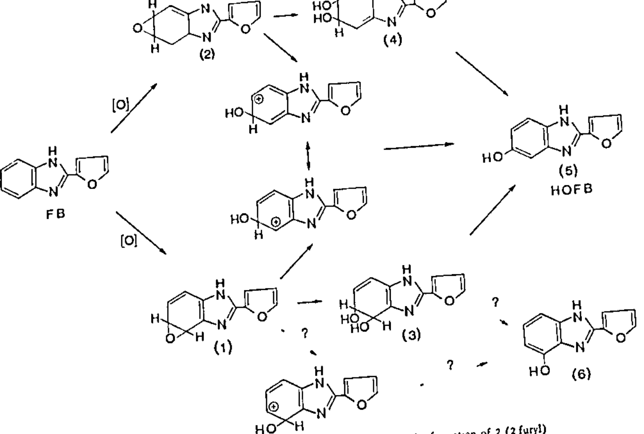
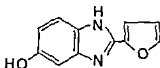


Diagram 2 — The scheme suggests a hydroxylation pathway in the formation of 2 (2 furyl) 5(6) hydroxybenzimidazole, metabolite Z.

The interpretation given is supported by the occurrence of a bathochromic shift in the UV spectrum of the metabolite on addition of alkali In accordance with its phenolic character the compound gives a positive reaction with Folin Ciocalteu reagent

The animal metabolism of certain benzimidazole derivatives, in which the benzene nucleus is unsubstituted, has previously been investigated As can be seen from these investigations the degradation products are 5(6) hydroxylated benzimidazole derivatives (Tocco *et al* 1964, Jones *et al* 1965, Gardiner *et al* 1968) According to Ito *et al* (1961), however, 4(7) hydroxylated benzimidazoles are also obtained in addition to the 5(6) hydroxylated compounds It would thus seem reasonable that also the metabolism of FB would result in 5(6) hydroxylated benzimidazole derivatives

For this reason 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB) was synthesized This compound proved to be identical with metabolite Z with respect to chromatographic properties, UV spectra in aqueous solutions at various pH values and IR spectra Hence the structural formula of metabolite Z is as follows



A mechanism of microsomal hydroxylation of aromatic substrates has been elucidated recently (Daly *et al* 1968) The enzymatic hydroxylation with known "mixed function oxidases might be accompanied by migration of hydrogen from the site of the hydroxylation to the adjacent position in the aromatic ring This phenomenon has been demonstrated by studies involving aromatic compounds labelled with a heavy hydrogen isotope (^2H, ^3H) A similar migration of a chlorine atom has also been reported The term NIH shift has been coined for this phenomenon In this type of hydroxylation the microsomal system acts as a weak selective electrophile

Jerina *et al* (1969) recently demonstrated that 1,2 naphthalene oxide is a primary intermediate in the microsomal oxidation of naphthalene This arene oxide can yield the corresponding dihydrodiol by microsomal hydration or mainly the 1 naphthol by rearrangement These authors suggested that other aromatic compounds as well may be hydroxylated microsomally in an analogous manner Holtzman *et al* (1967) have demonstrated in the enzymatic conversion of naphthalene to the corresponding dihydrodiol that whereas the first oxygen atom is taken from atmospheric oxygen, the second oxygen atom (in the 2 position) is derived from water

In a microbial oxidation of benzene and toluene the dihydrodiol is formed in a different way, in that both oxygen atoms of the dihydrodiol originate from atmospheric oxygen (Gibson *et al* 1970)

Chapter 6. Preparation of crude concentrates of urinary metabolites A, B and E

The analytical method described in Chapter 3 made it possible to detect the presence of a number of metabolites of FB in horse, goat, dog, rabbit, and rat urine, after oral administration of the compound Among the metabolites observed, three, denoted A, B and E, respectively, were present in each of the species examined For this reason metabolites A, B and E are considered to be the main metabolites in the mammalian metabolism of FB To accomplish an investigation of the FB metabolism, however, it was necessary to identify and characterize these main metabolites

The present chapter describes the isolation of contaminated concentrates of metabolites A, B and E from horse urine and of metabolite A from dog urine

EXPERIMENTAL

PRINCIPLE

Metabolite-containing urine was extracted with a mixture of organic solvents The extract was evaporated, and the residue dissolved in a buffer solution Column chromatographic fractionation of this solution yielded metabolite-containing eluates which were concentrated by evaporation

MATERIAL

Horse urine obtained in the experiment described in Chapter 3 on p 24 was used A male mongrel dog, weighing 10 7 kg and placed in a metabolism cage, received an oral dose of FB (910 mg) in gelatin capsules Urine (260 ml) collected during 24 h after the administration of FB was used in the preparation of crude concentrates Where turbidity occurred, the samples were centrifuged

EXTRACTION OF METABOLITES FROM URINE

The clear urine was extracted three times with an equal volume of a mixture containing ethyl acetate and *n* butanol *in the proportions* 1 1 *(by vol) The pooled organic extracts* were evaporated at *reduced* pressure in a rotary evaporator *at 40° The thick* residue from the urine was dissolved in ammonium hydrogen carbonate buffer *(0 1 M, pH 8) An* addition of *n* butanol (1 per cent V/V) had been made to the buffer to prevent microbial growth

The extract was stored at —20° prior to chromatographic processing

Daly *et al* (1970) recently reported a hydroxylation mechanism of aromatic compounds that differs from the above mentioned mechanism According to the mechanism reported by Daly the hydroxylation of aromatic compounds, using horseradish peroxidase as a catalyst, is not associated with any NIH shift The existence of the HO_2 radical has been demonstrated in this type of hydroxylation Knowing that this radical alone is not capable of hydroxylating aromatic compounds, the existence of an active "enzyme complexed radical" was postulated

It would seem most probable that the hydroxylation of FB in the animal organism occurs by the action of a microsomal enzyme system (according to the mechanism suggested by Daly *et al* 1968 and Jerina *et al* 1969) The possible course of the reaction may be seen in the tentative Diagram 2, p 55 The biochemical attack is believed to start with the formation of an arene oxide intermediate, either 4,5 (1) or 5,6 benzimidazole oxide (2) These compounds are believed to yield the phenols, (5) and (6), by rearrangement, or the corresponding dihydrodiols (3) and (4), following hydration by microsomal epoxide hydrase Compounds (3) and (4) on dehydration yield the hydroxylated benzimidazole derivative HOFB (5) According to this scheme the formation of a 4(7) substituted phenol (6) may also be expected, the presence of which, as well as of the suggested dihydrodiols (3) and (4), however, still remains to be demonstrated The scheme suggested for the metabolism of FB is corroborated by the hydroxylation induced migration of a chlorine atom in the metabolism of 4,5-dichloro 2 trifluoromethylbenzimidazole in the rat and in a microsomal NADH enzymatic system prepared from mouse liver homogenate, leading to the formation of 4,6 dichloro 5 hydroxy 2 trifluoromethyl benzimidazole (Bowker & Casida 1969)

SUMMARY

Metabolite Z has been isolated by chromatographic separation on a Sephadex column of urine from a goat that had been given FB orally The metabolite from the column eluates was concentrated by means of extraction and purified by thin layer chromatography Comparisons of the chromatographic properties in column and thin layer chromatography, the UV spectra in aqueous solutions at various pH values, and the IR spectra all proved metabolite Z and synthetic 2 (2 furyl) 5(6) hydroxybenzimidazole to be identical Evidence based on the NMR spectrum of the synthetic compound was used as the definite structural proof Metabolite Z is hence 2 (2 furyl) 5(6) hydroxybenzimidazole

dissolved in ammonium hydrogen carbonate buffer (0.1 M, 250 ml) and chromatographed in lots of 25 ml. The column eluates containing metabolites A, B and E were collected separately and evaporated. Each evaporation residue formed a concentrate of the metabolite concerned. This column chromatographic fractionation was repeated six times. The concentrates of metabolites A, B and E resulting from evaporating the eluates were collected and combined, each metabolite separately.

The concentrate of metabolite A was a thick and sticky material. The concentrate of metabolite B weighed 1.1 g and that of metabolite E 0.46 g. The latter two were brown powders.

Dog urine. — Following extraction and subsequent evaporation of the pooled extracts the centrifuged and clear urine (250 ml) yielded a brown and viscous residue that was dissolved in ammonium hydrogen carbonate buffer (0.1 M, 10 ml) and subjected to column chromatography. The column eluates containing metabolite A were collected and evaporated, resulting in a brown and viscid residue.

COMMENTS ON THE METHOD

In column chromatographic fractionation of horse urine (*cf.* Fig. 6, p. 29) metabolites A, B and E were found to be present in considerable amounts, suggesting that horse urine would offer a suitable raw material for the isolation of the three metabolites on a preparative scale. Since the structure of the metabolites was unknown it was considered important to apply procedure under mild conditions to reduce as far as possible the risk of hydrolysis or other structural changes during separation and isolation.

With the column chromatographic procedure involving Sephadex (Chapter 3) the separation of the metabolites was definitely carried out under mild conditions and it would hence have been desirable to apply the same separation technique on a larger scale. Unfortunately, the capacity of the column chromatographic procedure is limited, especially with respect to the sample volume, which made it necessary to concentrate the metabolites prior to the column chromatography.

In the present investigation the enrichment of the metabolites was effected by extraction of the urine by means of a mixture of organic solvents.

Extraction by organic solvents, such as *n*-butanol, is one of the procedures commonly used in the isolation of phenolic conjugates. In this procedure the extraction and the removal of the organic solvent are carried out rapidly. Since *n*-butanol dissolves considerable quantities of water as well, which may contribute to an increase of contaminants as inorganic salts in the organic phase, the extraction is performed with a mixture of *n*-butanol and ethyl acetate.

A small-scale extraction of metabolite-containing horse urine with subsequent column chromatographic separation according to Chapter 3 revealed that all of the metabolites, *i.e.* A, B (C) and E were extracted. This proved the possibility of performing extraction and fractionation by column chromatography on a preparative scale.

COLUMN CHROMATOGRAPHIC FRACTIONATION OF URINE EXTRACT

A glass column (5.5 cm i.d.) was packed with preswollen Sephadex G-25 gel, fine (Pharmacia) to a height of 100 cm. The pretreatment of the gel and the packing of the column were carried out as described previously (Chapter 3, p. 25) in connection with chromatography of urine samples. Urine extract ($\leq$25—30 ml) was transferred to the column and separated in a manner similar to that described for column chromatography (*cf.* Chapter 3). The UV transmission of the column eluate was recorded continuously at 280 nm by an LKB Uvicord II ultraviolet absorptiometer, using a 0.5 mm flow cell. The fractions (60 ml) were collected by means of a time-controlled automatic fraction collector. The separation process usually went on for 5—6 days.

REGENERATION OF COLUMN BED

Several compounds present in the urinary extract, some of them heavily coloured, were adsorbed very strongly on the Sephadex gel. For the purpose of regeneration the gel in the column was washed with a solution of ethanol in water (33 per cent V/V) until the packing material became colourless. During this procedure the column bed shrank by some 20 per cent of its length. When the ethanolic solution was replaced with aqueous buffer the gel tended to regain its original volume. This process was hindered by the narrowness of the column, which as a result became clogged. To facilitate the swelling procedure the gel was carefully agitated with the buffer. Subsequently the column was washed and equilibrated with the buffer to prepare it for repeated use. The ethanolic extract from the column washings was discarded.

EVAPORATION OF POOLED FRACTIONS

The fractions from the column chromatographic separations were examined by UV spectrophotometry in the manner described on p. 28. Fractions believed to contain the same metabolite, *i.e.*, belonging to the same peak on the UV curve (280 nm) and/or having identical UV spectra, were combined. The pooled fractions containing metabolites were evaporated individually at reduced pressure in a rotary evaporator at 40°. To decompose any ammonium hydrogen carbonate that had accompanied the buffer and was still present in the residue, the evaporation was repeated at least 3—4 times, after addition of a few ml of water. The concentrated material was stored at ambient temperature.

APPLICATION OF THE METHOD

Horse urine. — The centrifuged clear urine (5500 ml) was extracted, the pooled extracts then being evaporated as described above. The evaporation residue was

Chapter 7. Isolation and structural elucidation of metabolite B

Investigation of urine samples from various animal species following FB administration have disclosed the presence of a number of degradation products of FB One of these, characterized by $K_D \approx 4.1$ in column chromatography on Sephadex (Table 6 p 26) was observed in the urine of each test animal, in horse urine even in considerable amounts This degradation product is known as metabolite B in the present work (Chapter 3) Extraction of the horse urine with subsequent *fractionation by column chromatography* of the extract resulted in a heavily contaminated concentrate of metabolite B (Chapter 6) The present chapter describes the isolation of metabolite B from this raw material and structural elucidation of it

EXPERIMENTAL AND RESULTS

APPARATUS

The optical rotations were determined by means of a Perkin Elmer polarimeter, Model 141 using a 100 mm micro cell Single readings were reproducible to $\pm 0.002°$ according to the manufacturer

All other equipment used in the work associated with the present chapter has been described in Chapter 5

MATERIAL

The concentrate of metabolite B prepared from horse urine as described in Chapter 6 was used as starting material in the isolation of the pure compound

ISOLATION OF METABOLITE B FROM CRUDE CONCENTRATE FROM HORSE URINE

TLC separation of metabolite B

The isolation and separation of the metabolite from its contaminants were accomplished by TLC of the crude concentrate of metabolite B obtained from horse urine using solvent systems I II and III (Table 7, p 27) The metabolite appeared in the chromatograms as a blue spot on examination in UV light (254 nm) At 366 nm the spot was bluish violet The R_f values of the metabolite in the three solvent systems were 0 35 0 23 and 0 05 respectively On examination in UV

SUMMARY

The preparation of metabolite concentrates from urine samples, following administration of FB, is described The method comprises extraction of the urine with a mixture of organic solvents (*n* butanol ethyl acetate, 1 1, by vol) followed by evaporation of the pooled extracts and chromatographic fractionation on a Sephadex G 25 column On evaporation the metabolite containing column eluates yield the various metabolite concentrates

The procedure described resulted in a complete separation of the main metabolites A, B and E that had previously been concentrated by extraction The contaminated metabolite concentrates thus obtained were considered useful intermediates in the process of separation and isolation of the various metabolites

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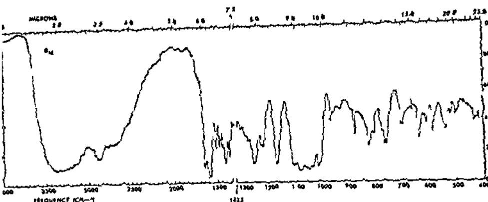


Fig 26 — IR spectrum of metabolite B in potassium bromide disk.

metabolite B were then re-chromatographed, using a column (25 mm i d) packed with equilibrated silica gel (250 g) to a bed height of 57 cm The eluates were divided into pre fraction main and residual fractions, which were evaporated individually in a rotary evaporator at reduced pressure and at room temperature The main fraction was concentrated to about 1 ml On keeping the solution at 0° over night, a precipitate was formed, which was collected by filtration and washed with a few drops of methanol Yield 20 mg from 1 1 g of crude starting material The product consisted of small pink needles, melting at 268—271°
$[\alpha]_D^{25} = -78\,6°$ $[M]_D^{25} = -310°$ (C 0 46, phosphate buffer, 0 2 M, pH 7 0) (Found C 51 8, H 4 3, N 6 9 $C_{17}H_{16}N_2O_8\,H_2O$ requires C 51 8, H 4 6, N 7 1)

UV spectrophotometric investigations were performed in accordance with the descriptions on p 46 The results are summarized in Table 11

The IR spectrum is illustrated in Fig 26 Strong absorption bands in the range 3700—2300 cm^{-1} indicate the presence of strong hydrogen bonds Complex bands at 1150—1000 cm^{-1} are attributed to vibrations of —C—O bonds of a sugar moiety The absorption at 1600 cm^{-1} is attributable to the antisymmetric stretching vibration of a carboxylate group The sharp band at 885 cm^{-1} indicates the intact furan nucleus

Table 11 — UV spectra of metabolite B in various solvents and at different pH values

Solvent	λ_{max} nm (log ε)			
ethanol abs	316	(4 47)	251	(4 09)
hydrochloric acid (0 01 M)	322	(4 51)	259	(4 05)
sodium hydroxide (0 01 M)	316	(4 48)	248	(4 18)
phosphate buffer (μ=0 1 pH 7 0)	314	(4 49)	251	(4 10)
ammonium hydrogen carbonate buffer (0 1 M pH 8 1)	314	(4 49)	251	(4 09)

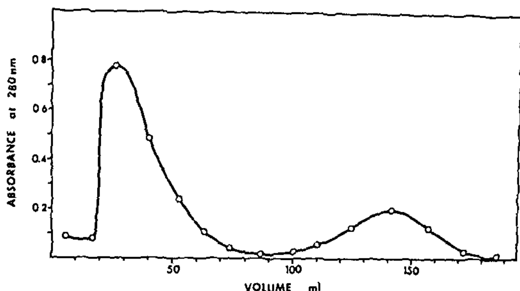


Fig 25 — *Purification of the crude concentrate of metabolite B by means of dry column chromatography* The naturally occurring impurities separate completely from metabolite B and leave the column first The UV absorbance of the eluates was evaluated at 280 nm

light (254 nm) there also appeared a spot, denoted as substance (x), with R_f= 0 43 0 40 and 0 23 in the solvent systems above This spot was close to that of metabolite B in the chromatograms

On spraying the chromatogram with naphthoresorcinol reagent (Stahl 1962) the spot containing metabolite B became reddish brown, and the spot corresponding to substance (*x*) became blackbrown No colouration occurred when the chromatogram was sprayed with Folin Ciocalteu reagent (Elliott 1959)

Dry-column chromatographic (DCC) separation and isolation of metabolite B

In DCC (*cf* Loev *et al* 1965, 1967) use was made of silica gel prepared for dry column chromatography (M Woelm, Eschwege, Ger) with solvent system II (Table 7, p 27) as eluent The eluent (10 ml) was added to silica gel (50 g) in a glass stoppered flask, which was agitated vigorously and then left for 10—12 h to allow the contents to equilibrate A glass column (20 mm i d) was packed with the equilibrated gel (height of bed 26 cm)

The crude concentrate of metabolite B (240 mg) was dissolved in methanol (5 ml) Silica gel (3 g) was added and the slurry was dried in a rotary evaporator at reduced pressure and at room temperature This material was placed on top of the column and eluted with the developing solvent The migration of metabolite B through the column was observed by illumination with long wave UV light The eluates were examined spectrophotometrically over the range 220—360 nm The separation is illustrated in Fig 25 Substance (*x*) left the column first quite in agreement with the R_f values from the TLC The eluates containing metabolite B were collected and evaporated in a rotary evaporator at reduced pressure and at the ambient temperature The pooled fractions from four separations containing

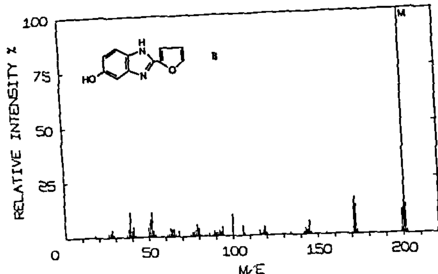


Fig 28 — Mass spectrum of metabolic 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB), obtained by acid hydrolysis of metabolite B

Determination of the aglycone (HOFB), following TLC (Method I) Five samples of the hydrolyzate (25 μl each) were chromatographed with solvent system I (Table 7, p 27), employing metabolite B and synthetic HOFB as references After development, on examination of the chromatograms in UV light two spots appeared in the hydrolyzate samples, one spot with $R_f=0\ 35$, corresponding to metabolite B, the other with $R_f=0\ 81$, corresponding to HOFB On spraying one plate with Folin Ciocalteu reagent, blue spots were observed only at $R_f=0\ 81$ Spots from each of the four separations were eluted individually with ethanol The content of metabolite B and HOFB was determined quantitatively by means of UV spectrophotometry The amount of unhydrolyzed metabolite B corresponded to 16 (16—17) per cent of metabolite B and the amount of hydrolyzed HOFB to 65 (62—68) per cent The UV absorption curve of the eluate corresponding to HOFB ($R_f=0\ 81$) proved to be identical with that of synthetic HOFB and showed the same bathochromic shift on addition of acid and alkali, respectively (*cf* Fig 21)

Determination of the aglycone (HOFB), following extraction (Method II) The remaining part of the hydrolyzate was diluted with water to 10 ml, 2 ml of which was transferred to a separating funnel This solution was neutralized with solid ammonium hydrogen carbonate (60 mg) and mixed with phosphate buffer (1 M pH 7 0, 1 ml) The solution was extracted three times with ethyl acetate (5 ml) The pooled organic extracts were washed with the phosphate buffer (1 ml) and extracted with hydrochloric acid (0 1 M, 20 ml) The HOFB content was determined quantitatively by means of UV spectrophotometry The amount of HOFB actually found was 66 per cent of the calculated value The quantitative

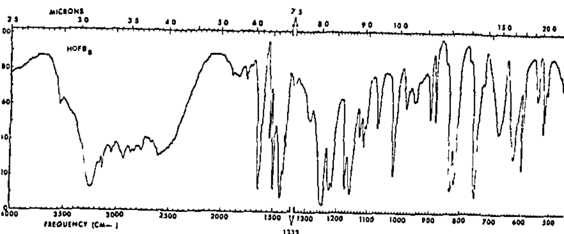


Fig 27 — IR spectrum of metabolic 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB) in potassium bromide disk, obtained by acid hydrolysis of metabolite B

HYDROLYSIS OF METABOLITE B

Preparative-scale hydrolysis with hydrochloric acid. Isolation of the aglycone (HOFB)

The residual fraction from the DCC separation was dissolved in hydrochloric acid (2 M, 5 ml) and boiled under reflux for 3 h During the hydrolysis the colour of the solution darkened and a precipitate appeared Following addition of charcoal, the solution was filtered The filtrate was neutralized with sodium hydroxide (2 M) and extracted five times with ethyl acetate (20 ml each) The organic phases were collected and dried over anhydrous sodium sulphate Evaporation in a rotary evaporator at reduced pressure and at room temperature yielded a crystalline residue that was recrystallized from acetonitrile A yellow, microcrystalline product was obtained on cooling Yield 4 5 mg, m p 263—265° (de comp) (No depression with synthetic 2-(2 furyl) 5(6) hydroxybenzimidazole) (Found C 66 0, H 4 2, N 13 8 $C_{11}H_8N_2O_2$ requires C 66 0, H 4 0, N 14 0)

The UV spectra in aqueous solutions at various pH values were identical with those of synthetic HOFB (*cf* Fig 21)

The IR spectrum (KBr), which is shown in Fig 27, coincides with the spectrum of synthetic HOFB (Fig 22)

The mass spectrum, illustrated in Fig 28, is identical with the mass spectrum of synthetic HOFB (Fig 23) The base peak is the molecular peak at *m/e* 200

Micro-scale hydrolysis with hydrochloric acid. Quantitative analysis of products of hydrolysis

Metabolite B (0 98 mg) was hydrolyzed with hydrochloric acid (2 M, 2 ml) in a sealed ampoule in a boiling water bath for 1 h After cooling, the content of phenol (HOFB) and glucuronic acid was determined

The phenol content was determined according to two methods (I, II)

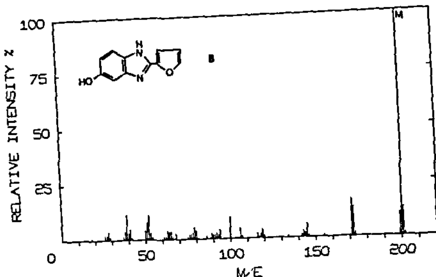


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Determination of the aglycone (HOFB), following TLC (Method I) Five samples of the hydrolyzate (25 μl each) were chromatographed with solvent system I (Table 7, p 27) employing metabolite B and synthetic HOFB as references After development, on examination of the chromatograms in UV light, two spots appeared in the hydrolyzate samples, one spot with R_f=0 35, corresponding to metabolite B, the other with R_f=0 81, corresponding to HOFB On spraying one plate with Folin Ciocalteu reagent, blue spots were observed only at R_f=0 81 Spots from each of the four separations were eluted individually with ethanol The content of metabolite B and HOFB was determined quantitatively by means of UV spectrophotometry The amount of unhydrolyzed metabolite B corresponded to 16 (16—17) per cent of metabolite B, and the amount of hydrolyzed HOFB to 65 (62—68) per cent The UV absorption curve of the eluate corresponding to HOFB (R_f=0 81) proved to be identical with that of synthetic HOFB and showed the same bathochromic shift on addition of acid and alkali, respectively (*cf* Fig 21)

Determination of the aglycone (HOFB), following extraction (Method II) The remaining part of the hydrolyzate was diluted with water to 10 ml, 2 ml of which was transferred to a separating funnel This solution was neutralized with solid ammonium hydrogen carbonate (60 mg) and mixed with phosphate buffer (1 M pH 7 0 1 ml) The solution was extracted three times with ethyl acetate (5 ml) The pooled organic extracts were washed with the phosphate buffer (1 ml) and extracted with hydrochloric acid (0 1 M, 20 ml) The HOFB content was determined quantitatively by means of UV spectrophotometry The amount of HOFB actually found was 66 per cent of the calculated value The quantitative

determination was carried out on two consecutive samples, the analytical results of which were identical

Determination of glucuronic acid The determination of glucuronic acid followed the method reported by Nir (1966), in which glucuronic acid reacts with naphthoresorcinol in a hot, strong hydrochloric acid solution After cooling to 0° the coloured reaction product was extracted with ethyl acetate The absorbance of the solution was determined by photometry at 580 nm The actual amount of glucuronic acid was calculated with the aid of a calibration curve

Of the previously diluted reaction solution 5 ml was diluted with an additional quantity of water to 10 ml The determination concerned here requires 2 ml The amount of glucuronic acid found was 99 per cent of the theoretical value (This figure is the mean of four determinations ranging between 97 and 101 per cent)

(Found $C_6H_9O_6$ 44 5 $C_{17}H_{16}N_2O_8$ H_2O requires $C_6H_9O_6$ 45 0)

Recovery of HOFB, following treatment with hydrochloric acid In the treatment of synthetic HOFB under the same conditions as mentioned under Micro scale hydrolysis with hydrochloric acid , 87 (81—91) per cent (employing TLC Method I) or 89 (88—90) per cent (with extraction Method II) of the added phenol (HOFB) could be accounted for In the former case four samples were examined, and in the latter case, two

Enzymatic hydrolysis using β-glucuronidase

A solution of metabolite B (0 90 mg) in phosphate buffer (0 2 M pH 7 0 0 2 ml) was transferred to each of three test tubes To the first one was then added bacterial β glucuronidase (1000 Fishman units of Sigma type II Sigma Chemical Co St Louis USA) dissolved in phosphate buffer (0 8 ml) as de scribed above To the second test tube was added enzyme that had been dissolved in the same manner except that it was heated at 100° for a few minutes prior to being added in the test tube To the third test tube phosphate buffer alone (0 8 ml) was added The tubes were plugged and incubated for 24 h at 38° Of the sample in each test tube 25 μl was transferred to thin layer plates and chromatographed with solvent systems I II and III (Table 7 p 27) using metabolite B and synthetic HOFB as references The detection revealed that the enzymatic hydrolysis was complete in test tube 1 *i e* only one spot could be *observed, the R_f value of which corresponded to that of synthetic HOFB* Controls 2 and 3 contained only metabolite B The aglycone spots on the three chromato plates were eluted with ethanol and the amounts were determined by means of UV spectrophotometry

The amount of aglycone present in the hydrolyzate was 88 (87—88) per cent of the theoretical value The UV spectra of the aglycone and the synthetic HOFB were identical in every respect (*cf* Fig 21) In TLC of the hydrolyzate applying co chromatography with synthetic HOFB and solvent system III only one spot could be detected

DISCUSSION

A comparison of the UV curves in Fig 6, recorded during the column chromatographic separation of samples of horse urine obtained before and after administration of FB, showed that the urine from the untreated animal contained a fraction exhibiting absorption in the UV that was eluted at the same speed as metabolite B The crude concentrate of metabolite B prepared from horse urine (Chapter 6) thus contained a considerable amount of contaminants It was possible, however, by means of TLC to isolate and separate the metabolite from this mixture The preparative separation was performed by DCC In order to avoid exposure of the metabolite to extreme pH values, the neutral solvent system II (*cf.* Table 7) was applied in this separation process Twenty mg of pure metabolite B was isolated from 1 1 g of the crude concentrate by means of repeated DCC. The compound, which was optically active (levorotatory), had the molecular formula $C_{17}H_{16}N_2O_8$ H_2O and melted at 268—271°.

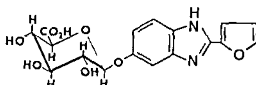
It may be assumed that metabolite B originates from FB by enzymatic action in the animal organism Presumably the benzene and/or the furan nucleus of FB is attacked in the course of metabolism The furan nucleus of the metabolite itself is intact, however, as may be concluded from the presence of an absorption band at 885 cm^{-1} in its IR spectrum

The UV spectrum of metabolite B (Fig 13, Table 11) has no fine structure but shows a maximum at 316 nm For the original compound (*i e*, FB) the corresponding maximum is at 307 nm This shift in wavelength would indicate that the benzene nucleus has been substituted by an electron donating group (*cf* Leandri *et al* 1955, Rabiger & Joullie 1964) For this reason metabolite B is believed to be a glucuronide of HOFB (*i e*, metabolite Z), with the molecular formula $C_{17}H_{16}N_2O_8$ H_2O This opinion is substantiated by the fact that metabolite B displays strong IR absorption bands (Fig 26) in the 3700—2300 cm^{-1} region, indicating that the compound contains hydrogen bonds The broad and intense absorption band at 3700—3100 cm^{-1} indicates the presence of water of crystallization Additional intense bands in the 1150—1000 cm^{-1} region suggest the presence of a sugar moiety (*cf* Barker *et al* 1956, Nakanishi 1962) The strong absorption band at 1600 cm^{-1} indicates a carboxylate group. From all of this evidence it may be inferred that metabolite B is a glucuronide with its carboxyl group ionized In agreement with this is the observation that the compound gives a positive reaction with naphthoresorcinol reagent

Glucuronides are formed in the metabolism of hydroxy compounds, carboxylic acids and some amino compounds in most mammalian species (Williams 1959, *d*) In the formation of a benzimidazole glucuronide both N- and O-conjugation may occur Although N-conjugation of benzimidazole (and of its derivatives) with ribose glucose or glucuronic acid has been reported (Kapoor & Waygood 1965, Bowker & Casida 1969), no such conjugation has taken place in this case as the compound does not show any bathochromic shift in its UV spectrum on alkalization Metabolite B may hence be assumed to be a 4(7)- or 5(6) O glucuronide

For reasons presented in connection with the structural elucidation of metabolite Z (Chapter 5, p 54), the 5(6)-O glucuronide structure would seem to be the more probable in the case of metabolite B In this case hydrolysis of metabolite B would yield the phenol HOFB

On hydrolysis with hydrochloric acid, metabolite B was found to yield a product with the molecular formula $C_{11}H_8N_2O_2$, mol wt 200, and m p 263—265°, which was identical in every respect with synthetic HOFB (Chapter 5, p 51) Acid hydrolysis on a micro scale of metabolite B yielded glucuronic acid and HOFB in a molar ratio of 1 1 HOFB was also observed on enzymatic splitting of metabolite B by β glucuronidase The strong substrate specificity of the enzyme suggests that the metabolite is a β glucuronide of HOFB This would also explain the strong levorotatory activity, a well known characteristic of β glucuronides (*cf* Williams 1959, *e*, Lemieux & Martin 1970) Summing up, metabolite B must be considered to be the β D glucopyranosiduronic acid derivative of 2 (2 furyl) 5(6) hydroxybenzimidazole with the structural formula



SUMMARY

Metabolite B is one of number compounds formed in the metabolism of FB The metabolite is present in the urine of all species investigated in the course of the present work It was isolated from a starting material obtained by column chromatographic fractionation of an extract from horse urine

Metabolite B is a glucuronide Acid hydrolysis of the metabolite yielded a phenol Evidence based on UV, IR and mass spectrometric data proved that this compound was identical *with the synthetically prepared compound* 2 (2 furyl) 5(6) hydroxy benzimidazole (HOFB)

Following acid hydrolysis, HOFB and glucuronic acid were determined quantitatively The molar ratio of the two compounds was calculated to be 1 1 Hydrolysis was also achieved by incubation with β glucuronidase

Evidence based on the IR spectrum together with other data proved the urinary metabolite B to be the β D glucuronide of 2 (2 furyl) 5(6) hydroxybenzimidazole

Chapter 8. Isolation and structural elucidation of metabolite E

A comparison of the UV curves in Fig 6, recorded during the column chromatographic separation of samples of horse urine obtained before and after administration of FB, showed that the latter contained a fraction, P_E ($K_D=5$ 5, *cf* Table 6, p 26), exhibiting absorption in the UV This fraction was believed to contain a metabolite, designated as metabolite E (Chapter 3) Fractionation by column chromatography of an extract of horse urine yielded a crude concentrate of this metabolite (Chapter 6) The concentrate was used subsequently as starting material in the isolation and structural investigation of metabolite E accounted for in the present chapter

EXPERIMENTAL AND RESULTS

MATERIAL

The concentrate of metabolite E recovered from horse urine as described in Chapter 6 was used as starting material in the isolation of the pure compound All equipment referred to in the present chapter has been mentioned previously (Chapter 5)

ISOLATION OF METABOLITE E FROM CRUDE CONCENTRATE FROM HORSE URINE

TLC separation of metabolite E

Applying TLC and solvent system IV (Table 7, p 27) it was possible to separate and isolate metabolite E from the contaminants present in the crude concentrate In the chromatograms metabolite E appeared as a light blue spot with $R_f=0$ 18 on examination in UV light at 254 nm In UV light at 366 nm the spot displayed a bluish white fluorescence Spraying with naphthoresorcinol or Folin Ciocalteu reagent did not produce any visible spots (*cf* Table 7)

DCC separation and isolation of metabolite E

In DCC according to Loev *et al* (1965, 1967) use was made of silica gel for dry column chromatography (M Woelm), with solvent system IV (Table 7) as eluent The eluent (17 5 ml) was added to the silica gel (120 g) in a glass-stoppered flask, which was agitated vigorously and then left for 10—12 h to allow the contents to equilibrate A glass column (25 mm i d) was packed with the equilibrated gel (height of bed 33 cm)

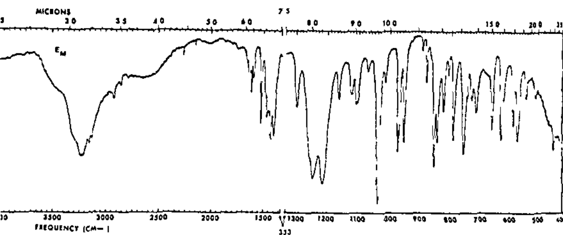


Fig 29 — IR spectrum of the ammonium salt of metabolite E in potassium bromide disk

The crude concentrate of the metabolite (107 mg) was dissolved in ammonium hydroxide (2 M, 1 ml) and water (4 ml) Silica gel (5 g) was added and the water was removed in a rotary evaporator at reduced pressure and ambient tem perature This material was placed on top of the column and eluted with the developing solvent The migration of metabolite E through the column was observed by examination in long wave UV light The eluates were examined spectrophoto metrically over the range 220—360 nm The eluates containing metabolite E were collected and evaporated under reduced pressure at 30° The residue was a white crystalline substance that could not be recovered by filtration owing to its strong hygroscopicity but was dissolved in a small amount of water Following addition of solid ammonium hydrogen carbonate (0 1 g) the solution was evaporated to dryness in a rotary evaporator A small amount of water was added to the residue and the resultant solution was re evaporated The evaporation and addition of water were repeated until no smell of ammonia was noticed The residue was dissolved in water (1 ml) On storage at 0° for 15 h a precipitate was formed which was collected by filtration The yield of this compound (α) was 15 3 mg (Found C 41 3, H 3 1, N 11 0, ash 8 5)

After keeping the filtrate at 0° for a number of days a precipitate appeared which was recovered by filtration The yield of this compound (β) was 8 mg[10] The product consisted of small pink needles that melted at 206—212° (decomp) (Found C 44 3, H 3 5, N 13 8 S 10 6 $C_{11}H_{11}N_3O_5S$ requires C 44 4 H 3 7 N 14 1, S 10 8)

UV spectrophotometric investigations of compound β were carried out according to the descriptions in Chapter 5 p 16 The results of these investigations are shown in Table 12

The IR spectrum (KBr) of compound β is illustrated in Fig 29 Absorption

[10] The filtrate related to compound β was kept for preparative scale hydrolysis with hydrochloric acid (see later in text)

Table 12 L V spect a f n etab l e L and sulpl ate ester of 2 (2 furyl) 5(6) l yd xybenz m dazole (HOFB) i var us sol ents and at d fferent pH values

S fvent	n etabol e E					sulpl ate ester of HOFB (synthet c)				
	λ ax n (l g ε)					λ ax nm (log ε)				
c l a l abs	32((1 37)	311 (4 48)	251 5	& 248	(4 09)	325 5 (4 3()	311 (4 48)	251 5	& 218	(4 08)
l yd icl l r c a d (0 01 M)		317 (4 55)	257 5		(4 08)		317 (1 53)	257 5		(4 01)
s d u n l yd x d (0 01 M)	327 5 (4 40)	313 (4 50)	247 5		(4 23)	327 5 (4 37)	313 (4 50)	247 5		(4 22)
pl spl ate buffer (μ) 1 pH 7 0)	324 (4 40)	309 (4 52)	250 5	& 247	(4 10)	324 (1 38)	30) (4 50)	250 5	& 247	(4 08)
a n un l yd ge i carb a e (0 1 M pH 8 1)	324 (4 4))	309 (4 52)	250 5	& 248	(4 10)	324 (4 38)	30) (4 50)	250 5	& 248	(4 08)

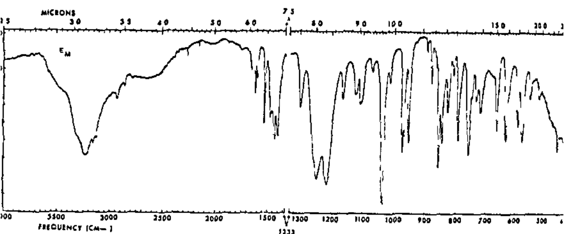


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[10] The filtrate related to compound β was kept for preparative scale hydrolysis with hydrochloric acid (see later in text)

Table 12 — UV spectra of metabolite L and sulphate ester of 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB) in various solvents and at different pH values

Solvent	metabolite E λ_{max} nm (*log e*)	sulphate ester of HOFB (synthetic) λ_{max} nm (log e)
ethanol abs	326 (4 37) 311 (4 48) 251 5 & 248 (4 08)	325 5 (1 36) 311 (4 48) 251 5 & 248 (4 08)
hydrochloric acid (0 01 M)	317 (4 55) 257 5 (4 08)	317 (4 53) 257 5 (4 04)
sodium hydroxide (0 01 M)	327 5 (4 40) 313 (4 50) 247 5 (4 23)	327 5 (4 37) 313 (4 50) 247 5 (4 22)
phosphate buffer (μ=0 1 pH 7 0)	324 (4 40) 309 (4 52) 250 5 & 247 (4 10)	321 (4 38) 309 (4 50) 250 5 & 247 (4 08)
ammonium hydrogen carbonate (0 1 M pH 8 1)	321 (1 40) 30) (1 52) 250 5 & 248 (4 10)	324 (4 38) 309 (4 50) 250 5 & 248 (4 08)

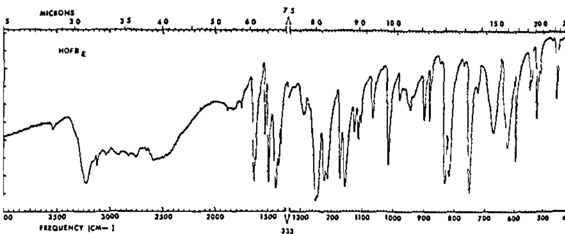


Fig 30 — IR spectrum of metabolic 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB) in potassium bromide disk obtained by acid hydrolysis of metabolite E

bands in the 3700—2200 cm^{-1} region are attributable to hydrogen bonds (probably to hydrogen bonded NH) The broad absorption band at 3230 cm^{-1} and the sharp band at 1405 cm^{-1} are probably caused by NH_4^+ The strong absorption bands at 1250, 1220 and 1042 cm^{-1}, the latter of which is the strongest in the spectrum are attributable to the sulphate ester group (Hearse *et al* 1969 Jerfy & Roy 1970) The absorption bands at 3160, 3130 and 888 cm^{-1} have been attributed to the furan nucleus (Wrobel & Galuszko 1970)

TLC investigations using the four solvent systems according to Table 7 on p 27 proved the substance to be homogeneous R_f values in the various solvent systems were in accordance with the values given in Table 7

HYDROLYSIS OF METABOLITE E AND SYNTHETIC SULPHATE ESTER OF HOFB

***Preparative-scale hydrolysis of metabolite E with hydrochloric acid* Isolation of HOFB**

The filtrate (0 8—0 9 ml) related to compound β as obtained in isolating metabolite E (see above) was mixed with hydrochloric acid (2 M 0 3 ml) and boiled for 5 min Bright yellow needles separated on cooling the solution The crystals were collected by filtration and washed with a few drops of hydrochloric acid (1 M) The product (17 mg) melted at 135—150° (decomp) (Found C 18 5 H 4 7, N 10 0 $C_{11}H_8N_2O_2$ HCl $2H_2O$ requires C 18 5, H 4 8 N 10 3)

UV spectra in aqueous solutions at different pH values were identical with the spectra of synthetic HOFB (*cf* Fig 21)

The IR spectrum (KBr) was identical with that of synthetic HOFB HCl $2H_2O$ (*i e*, compound IVa in Chapter 5)

The compound (11 5 mg) obtained from the hydrolysis of compound β was dissolved in water (*0 5 ml*) and *mixed* with ammonium hydrogen carbonate (1 M,

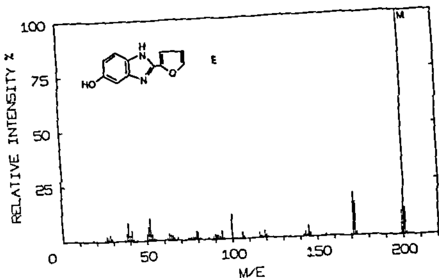


Fig 31 — Mass spectrum of metabolic 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB), obtained by acid hydrolysis of metabolite E

0 1 ml), resulting in a yellow precipitate that was washed with water *Following* recrystallization from acetonitrile the yield was 7 mg (83 per cent) The yellow substance melted at 260—262° (No depression with synthetic HOFB) (Found C 65 9 H 4 1, N 14 0 $C_{11}H_8N_2O_2$ requires C 66 0, H 4 0, N 14 0)

The UV spectra in aqueous solutions at different pH values were identical with those of synthetic HOFB (*cf* Fig 21, Table 10)

The IR spectrum (KBr), as shown in Fig 30, is in agreement with that of synthetic HOFB (Fig 22)

Fig 31 illustrates the mass spectrum, which is identical with the spectrum of synthetic HOFB (Fig 23) The base peak is the molecular peak M at *m/e* 200

Qualitative test for sulphate ion in acid hydrolyzate of metabolite E

A few drops of the filtrate related to compound β were mixed with one drop of barium chloride solution (0 5 per cent) before and after hydrolysis with hydrochloric acid In the latter solution there was an immediate cloud of turbidity, indicating the presence of sulphate ions One drop of water and one drop of the filtrate from compound β were evaporated on a filter paper prior to, and following, hydrochloric acid hydrolysis On spraying with barium chloride and sodium rhodizonate solutions a white spot appeared against an orange-red background only at the very place where the solution had been evaporated *on the filter* paper *after* the acid hydrolysis, indicating the presence of sulphate ions (*cf* Feigl 1954)

Micro scale hydrolysis with trichloroacetic acid (TCA)[11] Quantitative analysis of the products of hydrolysis

Hydrolysis of metabolite E (compound β)

Compound β (0 52 mg) was hydrolyzed in a TCA solution (4 per cent, 8 ml) in a sealed, thick walled glass ampoule during 12 h at 110° After cooling in the air the ampoule was further cooled with ice and opened carefully[12]

Determination of HOFB, following TLC (Method I) Two samples of the TCA hydrolyzate of compound β as described above (100 μl per sample) were chromatographed with solvent system II (Table 7, p 27) employing synthetic sulphate ester and synthetic HOFB as references Following development only one spot with $R_f = 0\ 74$, corresponding to HOFB appeared in each sample of hydrolyzate on examination in UV light The spots from two samples were eluted with ethanol for quantitative analysis by UV spectrophotometry The HOFB content was 88 per cent of the theoretical value in both samples

UV spectrophotometry of the eluates displayed the same bathochromic shift on addition of acid or alkali as stated previously for synthetic HOFB (cf Fig 21)

Determination of HOFB following extraction (Method II) Two samples of the hydrolyzate, each comprising 1 ml, were neutralized with solid sodium hydrogen carbonate and extracted with ethyl acetate (3×5 ml) The pooled organic extracts were washed with phosphate buffer (1 M, pH 7 0 1 ml) and subsequently extracted with hydrochloric acid (0 1 M, 10 ml) The HOFB content was determined by UV spectrophotometry The HOFB found was 87 per cent of the theoretical value The determination was carried out on two consecutive samples the analytical results of which were 86 and 87 per cent

The recovery of HOFB following TCA hydrolysis was investigated under experimental conditions that were identical with those applied in the hydrolysis of compound β The extraction method permitted recovery of 89 per cent of the HOFB added The determination was performed on duplicate samples the analytical results of which were identical

Determination of sulphate ion As a preliminary to quantitative determination of the sulphate content the aryl sulphate esters are hydrolyzed in a TCA solution (4 per cent) (Dodgson 1961) On mixing the hydrolyzate with a solution of barium chloride and gelatin turbidity occurs if sulphate ions are present The gelatin is to stabilize the barium sulphate suspension the turbidity of which is then read on a photometer at 500 nm The actual amount of sulphate is calculated with the aid of a calibration curve

The TCA hydrolyzate of compound β (4 ml) was mixed with barium chloride

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[11] Hydrolysis of aryl sulphate esters in TCA (cf Dodgson 1961)

[12] The ampoule must be opened with great care owing to the excess pressure caused by thermal degradation of TCA

gelatin solution (1 ml), the sulphate content being evaluated by means a photometer using a 20 mm cell, with reagent as a blank

(Found SO_4^{2-} 32.1. $C_{12}H_{12}N_3O_5S$ requires SO_4^{2-} 32.3.)

Hydrolysis of synthetic sulphate ester of HOFB. Determination of HOFB and sulphate ion

Synthetic sulphate ester of HOFB (0.95 mg) was hydrolyzed in TCA (4 per cent, 12 ml) as described for compound β. (In a TCA solution containing synthetic sulphate ester of HOFB at the same concentration as in the hydrolysis, no sulphate ions could be detected prior to heating.) TLC of the hydrolyzate proved the cleavage to be complete. The R_f value of the product formed was identical with the corresponding value of HOFB in solvent system II (*cf.* Table 7, p. 27). The quantitative determination of HOFB according to the extraction method yielded 86 per cent of the theoretical content. The determination was performed on two consecutive samples, the analytical results of which were identical. The sulphate content as well was determined on duplicate samples, the results of which were identical.

(Found SO_4^{2-} 29.7. $C_{11}H_7N_2O_5SNa \cdot H_2O$ requires SO_4^{2-} 30.0.)

Enzymatic hydrolysis of metabolite E (compound β) and synthetic sulphate ester of HOFB

Compound β (0.34, 0.29 and 0.31 mg) was added to three test tubes (1), (2) and (3), respectively. Synthetic sulphate ester (0.32, 0.28 and 0.30 mg) was weighed into another set of three test tubes (1'), (2') and (3'), respectively. Arylsulphatase[13] (13 *units/µmole substrate*) was added to each of the test tubes (1) and (1') dissolved in sodium acetate buffer (0.1 M, pH 5.0, 0.3 ml). The enzyme was also added to tubes (2) and (2') under the same conditions except that it was heated at 100° for a few minutes prior to addition. To test tubes (3) and (3') acetate buffer (0.3 ml) alone was added. The tubes were plugged and incubated during 40 h at 38°. Samples of 25 µl from each test tube were chromatographed using solvent systems II, III and IV (*cf.* Table 7, p. 27), and with HOFB and synthetic sulphate ester of HOFB as references.

Examination of the chromatograms in UV light showed that hydrolysis occurred only in test tubes (1) and (1'). Of each hydrolyzate two spots appeared whose R_f values corresponded to those of HOFB and HOFB sulphate ester (*cf.* Table 7), indicating that the hydrolysis was incomplete. Control tubes (2), (2'), (3) and (3') contained only starting material.

In TLC of the hydrolyzates applying co-chromatography with synthetic sulphate ester and synthetic HOFB and using solvent systems II, III and IV (*cf.* Table 7) the spots were homogeneous.

[13] Sulphatase obtained from limpets. Sigma type III (Sigma Chemical Co.). The activity was 8.3 units per mg according to the manufacturer. One unit hydrolyzes 1 µmole nitrocatechol sulphate per hour at pH 5.0 and 37°.

Micro scale hydrolysis with trichloroacetic acid (TCA)[11] Quantitative analysis of the products of hydrolysis

Hydrolysis of metabolite E (compound β)

Compound β (0 52 mg) was hydrolyzed in a TCA solution (4 per cent 8 ml) in a sealed thick walled glass ampoule during 12 *h at 110°* *After* cooling in the air the ampoule was further cooled with ice and opened carefully[12]

Determination of HOFB, following TLC (Method I) Two samples of the TCA hydrolyzate of compound β as described above (100 μl per sample) were chromatographed with solvent system II (Table 7, p 27) employing synthetic sulphate ester and synthetic HOFB as references Following development only one spot with R_f=0 74 corresponding to HOFB appeared in each sample of hydrolyzate on examination in UV light The spots from two samples were eluted with ethanol for quantitative analysis by UV spectrophotometry The HOFB content was 88 per cent of the theoretical value in both samples

UV spectrophotometry of the eluates displayed the same bathochromic shift on addition of acid or alkali as stated previously for synthetic HOFB (*cf* Fig 21)

Determination of HOFB following extraction (Method II) Two samples of the hydrolyzate each comprising 1 ml were neutralized with solid sodium hydrogen carbonate and extracted with ethyl acetate (3×5 ml) The pooled organic extracts were washed with phosphate buffer (1 M, pH 7 0 1 ml) and subsequently extracted with hydrochloric acid (0 1 M 10 ml) The HOFB content was determined by UV spectrophotometry The HOFB found was 87 per cent of the theoretical value The determination was carried out on two consecutive samples the analytical results of which were 86 and 87 per cent

The recovery of HOFB following TCA hydrolysis was investigated under experimental conditions that were identical with those applied in the hydrolysis of compound β The extraction method permitted recovery of 89 per cent of the HOFB added The determination was performed on duplicate samples the analytical results of which were identical

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The TCA hydrolyzate of compound β (4 ml) was mixed with barium chloride

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[11] Hydrolysis of aryl sulphate esters in TCA (*cf* Dodgson 1961)

[12] The ampoule must be opened with great care owing to the excess pressure caused by thermal degradation of TCA

gelatin solution (1 ml) the sulphate content being evaluated by means a photometer using a 20 mm cell, with reagent as a blank.

(Found SO_4^{2-} 32.1 $C_{11}H_{11}N_3O_5S$ requires SO_4^{2-} 32.3.)

Hydrolysis of synthetic sulphate ester of HOFB Determination of HOFB and sulphate ion

Synthetic sulphate ester of HOFB (0.95 mg) was hydrolyzed in TCA (4 per cent 12 ml) as described for compound β (In a TCA solution containing synthetic sulphate ester of HOFB at the same concentration as in the hydrolysis, no sulphate ions could be detected prior to heating.) TLC of the hydrolyzate proved the cleavage to be complete. The R_f value of the product formed was identical with the corresponding value of HOFB in solvent system II (*cf* Table 7, p 27). The quantitative determination of HOFB according to the extraction method yielded 86 per cent of the theoretical content. The determination was performed on two consecutive samples the analytical results of which were identical. The sulphate content as well was determined on duplicate samples, the results of which were identical.

(Found SO_4^{2-} 29.7 $C_{11}H_7N_2O_5SNa \cdot H_2O$ requires SO_4^{2-} 30.0.)

Enzymatic hydrolysis of metabolite E (compound β) and synthetic sulphate ester of HOFB

Compound β (0.34 0.29 and 0.31 mg) was added to three test tubes (1), (2) and (3) respectively. Synthetic sulphate ester (0.32 0.28 and 0.30 mg) was weighed into another set of three test tubes (1'), (2') and (3') respectively. Aryl sulphatase[13] (13 units/μmole substrate) was added to each of the test tubes (1) and (1') dissolved in sodium acetate buffer (0.1 M, pH 5.0, 0.3 ml). The enzyme was also added to tubes (2) and (2') under the same conditions except that it was heated at 100° for a few minutes prior to addition. To test tubes (3) and (3') acetate buffer (0.3 ml) alone was added. The tubes were plugged and incubated during 40 h at 38°. Samples of 25 μl from each test tube were chromatographed using solvent systems II III and IV (*cf* Table 7 p 27), and with HOFB and synthetic sulphate ester of HOFB as references.

Examination of the chromatograms in UV light showed that hydrolysis occurred only in test tubes (1) and (1'). Of each hydrolyzate two spots appeared whose R_f values corresponded to those of HOFB and HOFB sulphate ester (*cf* Table 7) indicating that the hydrolysis was incomplete. Control tubes (2) (2') (3) and (3') contained only starting material.

In TLC of the hydrolyzates applying co-chromatography with synthetic sulphate ester and synthetic HOFB and using solvent systems II III and IV (*cf* Table 7) the spots were homogeneous.

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[13] Sulphatase obtained from limpets Sigma type III (Sigma Chemical Co.). The activity was 8.5 units per mg according to the manufacturer. One unit hydrolyzes 1 μmole nitrocatechol sulphate per hour at pH 5.0 and 37°.

The spots in the hydrolyzate chromatograms were eluted with ethanol and their contents were determined by UV spectrophotometry The results are given in percentages of the total amount of substance observed (*i e*, per cent of the sum total of HOFB and sulphate ester of HOFB observed) The hydrolyzates from test tubes (1) and (1) appeared to contain 80 and 81 per cent HOFB together with 20 and 19 per cent, respectively, of the starting material

UV spectrophotometry of the eluates from spots corresponding to HOFB displayed the same bathochromic shift on addition of acid or alkali as stated previously for synthetic HOFB (*cf* Fig 21)

ISOLATION OF SULPHATE ESTER OF HOFB FROM CRUDE SYNTHETIC PRODUCT BY MEANS OF DCC

The separation of synthetic sulphate ester from a crude product[14] was carried out by means of DCC in the manner previously described on p 69 for the separation of metabolite E

A column (20 mm i d) was packed with silica gel (67 g) that was equilibrated with the eluent, solvent IV (Table 7) (Bed height, 28 cm)

Crude product (148 mg) was dissolved in methanol (2 ml), silica gel (3 g) was added, and the solvent was removed in a rotary evaporator at reduced pressure and room temperature The material thus obtained was placed on top of the column and developed with the solvent The eluates containing the sulphate ester were collected and evaporated

The white, hygroscopic residue was dissolved in water (3 ml), sodium hydroxide solution (0 1 M) being added to attain pH 9 0 The solution was evaporated to dryness and the residue was treated with acetonitrile yielding white crystals that were recovered by filtration and washed with acetonitrile Yield 40 mg corresponding to 25—27 per cent of the crude product which was expected to contain approximately 33 per cent of the desired substance (judging from a nitrogen analysis) The colourless crystals melted at 265—270° (decomp) (Found C 40 9, H 2 7, N 8 5, S 10 1, ash 23 7 $C_{11}H_7N_2O_5SNa\,H_2O$ requires C 41 3 H 2 8, N 8 8, S 10 0, ash[15] 23 5)

UV spectrophotometric investigations were carried out according to the technique described in Chapter 5, p 46 The results are summarized in Table 12 p 71

The IR spectrum (KBr) is shown in Fig 32 This spectrum is almost identical with the corresponding spectrum of compound *β* the differences being caused by the presence of water of crystallization (absorption bands in the 3600—3100 cm^{-1} range), other crystal forms and the absence of NH_4^+ The strong and characteristic absorption bands of the sulphate ester group are located at 1260 and 1225

[14] The crude product of sulphate ester was synthesized from HOFB by Dr H Fex AB Leo Helsingborg Sweden

[15] Ash calculated as sodium sulphate (Na_2SO_4)

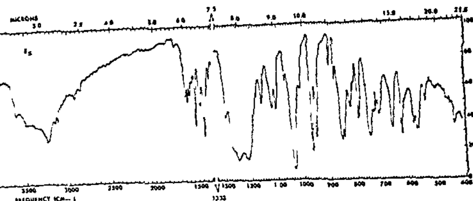


Fig 32 — IR spectrum of the synthetic sodium salt monohydrate of the sulphate ester of 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB) in potassium bromide disk

cm⁻¹ the strongest band in this spectrum, as in that of compound β (*cf* p 72) being found at 1046 cm⁻¹ The absorption bands at 3160, 3140 and 886 cm⁻¹ have been attributed to the furan nucleus (Wrobel & Galuszko 1970)

The substance was proved to be homogeneous by TLC tests, using the four solvent systems according to Table 7 The R_f values in the various solvents were the same as those characterizing metabolite E (*cf* Table 7) In column chromatography on Sephadex the synthetic product appeared to have the same K_D value ($K_D = 5.5$) as metabolite E

DISCUSSION

Applying TLC it was possible to separate and isolate metabolite E from the crude product obtained by column chromatographic fractionation of horse urine extract The preparative separation was performed by DCC From the crude concentrate (107 mg) two substances were isolated, one substance denoted α (15 3 mg) and the other β (8 mg) During combustion in connection with the elementary analysis compound α yielded ash whereas compound β left no traces The latter substance which contained sulphur proved to have the molecular formula $C_{11}H_{11}N_2O_5S$ and m p 206—212° The further investigation was carried out on compound β

There is a similarity between the UV spectrum of metabolite E (Fig 16, Table 12) and that of FB (Fig 2) The fine structure reveals less details than that of FB however In ethanol the spectrum has absorption maxima at 326 and 311 nm The corresponding maxima of FB are at 322 and 307 nm These findings suggest that a conversion has taken place in the FB molecule in the course of metabolism The benzene nucleus may be expected to be the site of the metabolic attack in the formation of metabolite E from FB This view is supported by the fact that the furan nucleus of metabolite E remains intact, which may be concluded from the

occurrence of absorption bands at 3160, 3130 and 888 cm 1 in the IR spectrum of compound β (Fig 29) (cf Wrobel & Galuszko 1970) After administration of FB, metabolites E and B have both been detected in urine from each of the animal species studied in the present work It has been established previously that metabolite Z is 2 (2 furyl) 5(6) hydroxybenzimidazole (Chapter 5) and that metabolite B is the O glucuronide of this phenol (Chapter 7) In view of the fact that phenols are also metabolized to sulphuric acid esters in the animal organism (cf Williams 1959, c), it would seem probable that the isolated metab olite E, compound β, may be an ammonium salt of the sulphate ester of the phenol HOFB A compound of this description should have the molecular formula $C_{11}H_{11}N_3O_5S$ (The ash remaining on combustion of substance α, together with the result of the elementary analysis, would suggest that the substance is a mixture of ammonium and alkali, probably sodium, salts of metabolite E) This view is further substantiated by the IR spectrum of the isolated metabolite E, which has intense absorption bands at 1250, 1220 and 1042 cm 1, indicating that a sulphate ester group is present (cf Hearse *et al* 1969, Jerfy & Roy 1970) This evidence together with the fact that the aqueous solution of the substance gives a positive reaction for sulphate ions with barium chloride and sodium rhodizonate only *after* boiling with hydrochloric acid, supports the idea that metabolite E is a sulphate ester Aliphatic and aromatic amines are also known to form sulphuric acid con jugates when metabolized in the mammalian organism (cf Williams 1959, c) In the present case, however, an N conjugation of the benzimidazole derivative does not occur, which can be concluded from the fact that the compound shows no bathochromic shift in the UV spectrum on alkalization

As has been mentioned previously in connection with the structural elucidation of metabolite Z (p 54), 5(6) hydroxylated benzimidazole derivatives have been observed in the metabolism of a number of benzimidazole, derivatives in which the benzene nucleus has not been substituted For this reason and since metabolites Z and B are both 5(6) substituted FB derivatives it would seem most probable that metabolite E as well would be a 5(6) substituted derivative of FB and more precisely a sulphate ester of the phenol HOFB This view being correct hydrolysis of metabolite E should yield the phenol HOFB

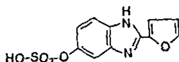
On hydrolysis with hydrochloric acid the isolated metabolite E was found to yield a substance with the molecular formula $C_{11}H_8N_2O_2$ mol wt 200 m p 260—262°, which was identical in every respect with synthetically prepared HOFB

On micro scale hydrolysis of the isolated metabolite E with trichloroacetic acid HOFB and sulphuric acid were obtained in the ratio of 1 1 Enzymatic cleavage of the isolated metabolite E with aryl sulphatase also yielded HOFB The marked substrate specificity of this enzyme offers further evidence that metabolite E is an aryl sulphate ester

From this it may be concluded that metabolite E is a sulphate ester of 2 (2 furyl) 5(6) hydroxybenzimidazole and that the isolated compound β is the ammonium salt of this compound

Further evidence for this structure was obtained by comparing the UV and IR spectra as well as the chromatographic properties of the isolated metabolite E and the completely synthetic sulphate ester of HOFB Also the hydrolytic properties of the synthetic product when treated with acid or with aryl sulphatase were identical with those of the isolated metabolite

Summing up, the structural formula of metabolite E is as follows



SUMMARY

Metabolite E is one of a number of degradation products formed by metabolism of FB in the mammalian organism It is present in the urine of each animal species investigated The pure compound was isolated by dry column chromatography from a crude concentrate obtained by chromatographic fractionation of an extract of horse urine

It was demonstrated that metabolite E is a sulphate ester of a phenol This phenol was prepared by acid hydrolysis of the isolated metabolite and identified as 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB) The identification is based on UV IR spectrophotometric mass spectrometric and other data obtained by investigation of the phenol and synthetic 2 (2 furyl) 5(6) hydroxybenzimidazole

Incubation of metabolite E as well as of synthetic sulphate ester of HOFB with aryl sulphatase resulted in incomplete hydrolysis The acid hydrolysis of the two substances by trichloroacetic acid however was complete and permitted quantitative determination of HOFB and SO_4^2 present in the hydrolyzates The molar ratio of these components was calculated to be 1 1

Evidence based on UV and IR spectra and other data shows that the isolated urinary metabolite E is the ammonium salt and the synthetic compound is the sodium salt monohydrate of the sulphate ester of 2 (2 furyl) 5(6) hydroxybenzimidazole

occurrence of absorption bands at 3160, 3130 and 888 cm 1 in the IR spectrum of compound β (Fig 29) (cf Wrobel & Galuszko 1970) After administration of FB, metabolites E and B have both been detected in urine from each of the animal species studied in the present work It has been established previously that metabolite Z is 2 (2 furyl) 5(6) hydroxybenzimidazole (Chapter 5) and that metabolite B is the O glucuronide of this phenol (Chapter 7) In view of the fact that phenols are also metabolized to sulphuric acid esters in the animal organism (cf Williams 1959, c), it would seem probable that the isolated metab olite E, compound β, may be an ammonium salt of the sulphate ester of the phenol HOFB A compound of this description should have the molecular formula $C_{11}H_{11}N_3O_5S$ (The ash remaining on combustion of substance α, together with the result of the elementary analysis, would suggest that the substance is a mixture of ammonium and alkali, probably sodium, salts of metabolite E) This view is further substantiated by the IR spectrum of the isolated metabolite E, which has intense absorption bands at 1250, 1220 and 1042 cm 1, indicating that a sulphate ester group is present (cf Hearse *et al* 1969, Jerfy & Roy 1970) This evidence together with the fact that the aqueous solution of the substance gives a positive reaction for sulphate ions with barium chloride and sodium rhodizonate only *after* boiling with hydrochloric acid, supports the idea that metabolite E is a sulphate ester Aliphatic and aromatic amines are also known to form sulphuric acid con jugates when metabolized in the mammalian organism (cf Williams 1959 c) In the present case however, an N conjugation of the benzimidazole derivative does not occur which can be concluded from the fact that the compound shows no bathochromic shift in the UV spectrum on alkalization

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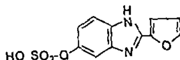
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Summing up the structural formula of metabolite E is as follows



SUMMARY

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Incubation of metabolite E as well as of synthetic sulphate ester of HOFB with aryl sulphatase resulted in incomplete hydrolysis The acid hydrolysis of the two substances by trichloroacetic acid however was complete and permitted quantitative determination of HOFB and SO_4^{2} present in the hydrolyzates The molar ratio of these components was calculated to be 1 1

Evidence based on UV and IR spectra and other data shows that the isolated urinary metabolite E is the ammonium salt and the synthetic compound is the sodium salt monohydrate of the sulphate ester of 2 (2 furyl) 5(6) hydroxybenzimidazole

Chapter 9. Isolation and structural elucidation of metabolite A

Metabolite A is one of the main products of the metabolism of FB in the mammalian organism This metabolite, which was observed by UV spectrophoto metry of the eluates from chromatography of metabolite containing urine on Sephadex columns, was characterized by $K_D = 2\ 0$ (Table 6, p 26) It was present in the urine of each animal species studied in the present investigation, after administration of FB (Chapters 3, 4) A crude concentrate of metabolite A was obtained by column chromatographic fractionation of extract from horse urine (Chapter 6) This concentrate was used as a starting material in the isolation and structural elucidation reported in this chapter Metabolite A has also been isolated from dog urine (*cf* Chapter 6)

EXPERIMENTAL AND RESULTS

APPARATUS

All equipment used in the work reported in the present chapter has been mentioned in Chapters 5 and 7 Circular dichroism (CD) measurements were performed on a Jouan Dichrograph, Model 185 When not otherwise stated, methanol was used as solvent in the CD investigations

MATERIAL

Concentrates of metabolite A from horse and dog urine as described in Chapter 6 were used as starting material in the isolation of the pure compound

ISOLATION OF METABOLITE A

Isolation from crude concentrate from horse urine

Concentrate from horse urine was dissolved in water (10 ml) Brown crystals appeared when the solution was kept at 0° The crystals were collected by filtration after one week and washed with a few ml of water Yield 150 mg

360 mg of the isolated compound was dissolved in a mixture (35 ml) of methanol and water in a ratio of 2 1 (by vol) The solution was concentrated to 20 ml charcoal was added, and the mixture was filtered while warm Light yellow crystals appeared on cooling and were recovered by filtration Yield 200 mg

Isolation from crude concentrate from dog urine

The separation was performed by means of TLC Thin layer plates (20×20 cm) were prepared with silica gel GF_{254} (E Merck) at a wet layer thickness of 0 5 mm as described in Chapter 3 p 26

The metabolite concentrate from dog urine (Chapter 6) was dissolved in ammonium hydrogen carbonate buffer (0 1 M, 2 ml) and applied as a band to the thin layer plates After development, using solvent system I (Table 7, p 27) the area of the silica gel on each plate corresponding to metabolite A was scraped off and eluted with warm methanol The filtered eluate was evaporated in a rotary evaporator at room temperature yielding 48 mg of slightly coloured crystals

COLUMN CHROMATOGRAPHIC PURIFICATION OF METABOLITE A

The subsequent purification of the compound was effected by chromatography on a Sephadex column (11 mm i d) using ammonium hydrogen carbonate buffer (0 1 M) in accordance with the separation technique applied to metabolites (Chapter 3 p 25)

The compound (20—30 mg) was dissolved in ammonium hydrogen carbonate buffer (0 1 M 1—2 ml) a few drops of ammonium hydroxide solution (2 M) being added to facilitate dissolution of the compound The solution was then chromatographed on the Sephadex column

The column eluates containing metabolite A were evaporated in a rotary evaporator at 30° the evaporation procedure being repeated following addition of water (1 ml each time) until no smell of ammonia could be noticed The final product was crystalline and colourless

The products isolated from horse and dog urine concentrates were homogeneous according to TLC using solvent systems I II and III All the R_f values were as stated in Table 7

PAPER ELECTROPHORESIS OF METABOLITE A

The paper electrophoresis was performed in an LKB 3276B apparatus The samples containing metabolite A were applied to standard paper strips (4 cm wide) of Schleicher & Schull type 2043b The strips were subjected to electrophoresis (300—320 V 6 h) in buffers at various pH values and a constant ionic strength $\mu = 0\ 1$ (Bloemendal 1963) On terminating the run the paper strips were examined in UV light (254 nm) and the light absorbing zones outlined The migration of the compound from the load line in the anodic (+) and cathodic (—) directions can be seen in Table 13

CHARACTERIZATION AND SPECTROSCOPIC INVESTIGATION OF METABOLITE A

The compound from *horse urine* melted at 206—210° (decomp) on a Kofler micro hot stage preheated to 185° $[\alpha]_D^{25} = -31\ 5°$ $[M]_D^{25} = -69\ 4°$ (C 0 53 hydrochloric acid 1 M) (Found C 60 0 H 5.5 N 12 7 $C_{11}H_{12}N_2O_3$ requires C 60 0 H 5 5 N 12 7)

Table 13 — Paper electrophoretic migration of metabolite A at different pH values (+) anodic migration, (—) cathodic migration

Buffer ionic strength μ=0 1	pH	distance from start cm
tris hydrochloric acid	8 0	+11 5
phosphate	7 1	+10 5
phosphate	6 1	+ 7 9
acetate	5 1	+ 6 0
acetate	4 0	— 0 5
citrate	3 0	— 2 4
glycine hydrochloric acid	2 0	— 2 8

The compound from *dog urine* melted at 206—210° (decomp) on a Kofler micro hot stage preheated to 185° $[\alpha]_D^{25}$ =—31 4°, $[M]_D^{25}$ =—69 1° (C 0 52, hydrochloric acid, 1 M) (Found C 59 8, H 5 4, N 12 6 $C_{11}H_{12}N_2O_3$ requires C 60 0, H 5 5, N 12 7)

The UV investigations were performed according to the descriptions on p 46 Figs 12 and 37 (f) illustrate the UV spectra in ammonium hydrogen carbonate buffer and absolute ethanol, respectively The UV absorption spectra of the com pounds from horse and dog urine are identical Table 14 shows absorption charac teristics of the compound isolated from horse urine established by measurements in various solvents

The IR spectrum (KBr) of metabolite A as isolated from horse urine is shown in Fig 33 Strong absorption bands in the 3600—1700 cm $^{-1}$ range indicate the presence of strong intermolecular and/or intramolecular hydrogen bonds Ab sorption bands at 3400 and 3150 cm $^{-1}$ are caused by stretching vibrations of hydrogen bonded OH and NH groups whereas the bands at 2960 2940 and 2920 cm $^{-1}$ are attributable to aliphatic C H stretching vibrations The strongest band in the spectrum is at 1560 cm $^{-1}$ which is assigned to the antisymmetric stretching vibration of a carboxylate anion (Bellamy 1958 Morgan 1961) The compounds from the horse and dog displayed identical IR spectra

The mass spectra of the compounds from the two different sources were also identical Fig 34 illustrates the mass spectrum of metabolite A as isolated from horse urine The molecular peak M is at *m/e* 220 other peaks of importance to

Table 14 — UV spectra of metabolite A in various solvents and at different pH values

Solvent	λ_{max} nm (log ε)					
ethanol abs	281	(3 83)	274	(3 81)	244 5	(3 72)
hydrochloric acid (0 01 M)	276	(4 02)	269 5	(3 99)	239	(3 61)
sodium hydroxide (0 01 M)	280	(3 86)	273	(3 87)	244	(3 75)
phosphate buffer (μ=0 1 pH 7 0)	280	(3 84)	273	(3 87)	244	(3 77)

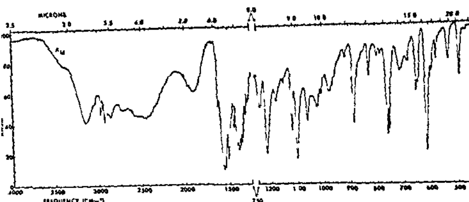


Fig 33 — IR spectrum of metabolite A in potassium bromide disk

the identification of the compound are the ion at *m/e* 202 (=M 18), the base peak at *m/e* 147 (=M 73) and the fragment ions at *m/e* 161 (=M 59), 148 (=M 72) 119 (=M 73 28) and 118 (=M 73 29) The fragmentation of the compound is discussed in Chapter 10

The NMR spectrum is shown in Fig 35 Metabolite A (70 mg) from horse urine was dissolved in DMSO d_6 (0 4 ml) and the spectrum run at 60 MHz The spectrum displays a multiplet in the region δ=1 7—2 7 (4H, CH_2 CH_2), a broadened triplet at δ=4 83 (1H, >CH O), an almost symmetrical multiplet centred at δ=7 26 (AA BB spectrum 4H, aromatic protons), and a very broad singlet at δ=9 07 (3H >N H O H CO O H) The position and shape of the last signal depend on the concentration Following dilution with DMSO d_6, it moves upfield developing into a very broad peak that overlaps the aromatic

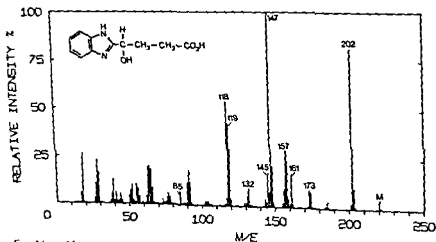


Fig 34 — Mass spectrum of metabolite A

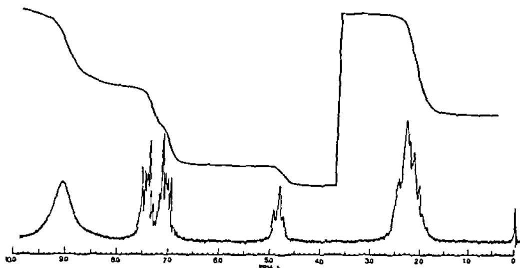


Fig 35 — NMR spectrum (60 MHz) of metabolite A in hexadeuterodimethylsulphoxide

multiplet at $\delta = 7\ 26$ On addition of a drop of D_2O the peak at $\delta = 9\ 07$ disappears and a sharp peak (DHO) appears at $\delta = 4\ 63$

The CD spectrum of metabolite A isolated from horse urine is shown in Fig 37 (e)

$\lambda_{max} = 279,\ 243,\ 213$ nm, $\Delta\varepsilon = +0\ 48,\ -1\ 52,\ +1\ 96$

SYNTHESIS OF (S) (—) 4 (2 BENZIMIDAZOLYL) 4 HYDROXYBUTYRIC ACID

The synthesis is depicted in Diagram 3 (*S*) (+) 4 chloroformyl γ butyrolactone[16] (*III*) was reacted with 2 nitroaniline, yielding the corresponding 2 nitrophenyl amide (IV) This was converted to 2 aminophenyl amide (V) by catalytic hydrogenation with palladium on carbon Cyclization of compound (V) was effected with simultaneous cleavage of the lactone ring by boiling the compound in dilute hydrochloric acid the result being (*S*) (—) 4 (2 benzimidazolyl) 4 hydroxybutyric acid (VII)

Cyclization of compound (V) in an anhydrous medium containing TCA (*cf* Meth Cohn & Naqui 1967) yielded (*S*) (+) 4 (2 benzimidazolyl) γ butyrolactone (VI)

Compound (III) was prepared from (*S*) (+) glutamic acid (I) that was deaminated (Pfleninger *et al* 1961) with retention of the configuration (Austin & Howard 1961, Cervinka & Hub 1968) yielding (*S*) 4 carboxy γ butyrolactone (II), which was then converted into compound (III) by thionyl chloride

From compound (IV) an optically active substance and a racemic mixture were obtained by means of fractional crystallization It was calculated from the specific rotation of the isolated enantiomer that the crude product of compound (IV) contained approximately 84 per cent enantiomer with *S* configuration The occurrence

[16] All substituted γ butyrolactones in the present investigation are numbered as derivatives of *butyric acid*

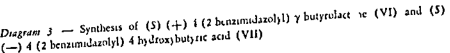


Diagram 3 — Synthesis of (*S*) (+) 4 (2 benzimidazolyl) γ butyrolactone (VI) and (*S*) (—) 4 (2 benzimidazolyl) 4 hydroxybutyric acid (VII)

of the *R* configuration suggests a certain inversion during the synthesis Similar results have been noted in the deamination of γ-aminovaleric acid (Moll 1966)

As support for this idea it may be mentioned that the specific rotation after repeated recrystallization of compound (II) was greater than the value reported for this compound in the literature (Červinka & Hub 1968) A calculation based on this difference indicates that the ratio between *S*- and *R* configuration apparently was of the same order of magnitude for the compound (II) mentioned in the literature (*loc cit*) as for the compound obtained by deamination in the present work

(S)-4-carboxy-γ-butyrolactone (II)

The starting material was (*S*) (+) glutamic acid (I) (min 99 per cent L (+)-glutamic acid as stated by the manufacturer, E Merck) $[\alpha]_D^{25} = +30\ 9°$ (C 1 0, hydrochloric acid, 6 M) Compound (I) was deaminated according to the standard procedure (Plieninger *et al* 1961)

A portion of compound (II) was dissolved in warm diethyl ether After 10—12 h at —20° crystals appeared, that were recovered by filtration After three recrystallizations from diethyl ether, the m.p of the crystals was 71—72° $[\alpha]_D^{22} = -4\ 0°$ (C 5 1, water), $[\alpha]_D^{22} = +14\ 2°$ (C 5 6, methanol)

CD spectrum $\lambda_{max} = 214$ nm, $\Delta\varepsilon = +1\ 56$

Lit (Červinka & Hub 1968) m p 71—72°, $[\alpha]_D^{22} = -2\ 8°$ (C 5 8 water), $[\alpha]_D^{22} = +10\ 6°$ (C 5 0, methanol)

The ORD curve exhibits a positive Cotton effect and an intersection point with the axis of zero rotation at 219 nm

(S)-(+)-4-chloroformyl-γ-butyrolactone (III)

The distillate from compound (II) was used without recrystallization in the preparation of compound (III) according to the method described by Plieninger *et al* (1961), and thus it was optically impure

$[\alpha]_D^{22} = +45\ 0°$ (C 0 5, benzene)

CD spectrum (cyclohexane) $\lambda_{max} = 250\ \ 188$ nm, $\Delta\varepsilon = +0\ 13\ \ +0\ 30$

(S)-(+)-N-(2-nitrophenyl)-4-carbamoyl-γ-butyrolactone (IV)

2 nitroaniline (1 4 g) and compound (III) (1 5 g) were heated in benzene (5 ml) until no more hydrochloric acid was developed (about 20—30 min), after which an additional quantity of benzene (5 ml) was added A yellow precipitate separated on cooling The crystals were recovered by filtration and washed with a small amount of benzene Yield 2 2 g (87 per cent) Following concentration of the filtrate, an additional quantity of the substance (0 2 g) was obtained from the mother liquor, bringing the overall yield to 95 per cent The product consisted of crystals of two shapes, *viz*, bright yellow needles and yellow prisms It melted at 110—120° $[\alpha]_D^{22} = +49\ 1°$ (C 0 5, chloroform)

This experiment was repeated with a batch that was 10 times as large as the one just mentioned By fractional crystallization from benzene the synthetic product was partitioned into a light yellow fraction (needles, 11 g), denoted A, and a yellow fraction (prisms, 6 g), denoted B In benzene solution, fraction B was the one to precipitate first

Fraction A — After several recrystallizations from benzene, the light yellow *needles melted at* 115—117° $[\alpha]_D^{22} = +71\ 2°$ (C 0 50, chloroform) (Found C 52 9, H 4 0, N 11 4 $C_{11}H_{10}N_2O_5$ requires C 52 8, H 4 0, N 11 2)

UV spectrum, ethanol λ_{max}=339, 234 nm, *log* ε=3 48 and 4 28, $\lambda_{shoulder}$ =268 nm, log ε=3 68

IR spectrum (KBr) doublet, 3300 cm 1 (amide NH), 1680 cm 1 (amide CO), 1790 and 1770 cm 1 (γ-lactone CO)

Fraction B — After several recrystallizations from benzene, the yellow prisms melted at 129 5—130 5 $[\alpha]_D^{22}=0°$ (C 0 50, chloroform) (Found C 53 0, H 3 9 N 11 4 $C_{11}H_{10}N_2O_5$ requires C 52 8, H 4 0, N 11 2)

UV spectrum, ethanol λ_{max}=342 and 236 nm, *log* ε=3 52 and 4 29, $\lambda_{shoulder}$ =266 nm, log ε=3 69

IR spectrum (KBr) 3300 cm 1 (amide NH), 1690 cm 1 (amide CO), 1780 cm 1 (γ lactone CO)

The mass spectra of fractions A and B are identical

(S)-(+) N-2 (aminophenyl)-4-carbamoyl-γ-butyrolactone (V)

Compound (IV) (1 9 g) and 5 per cent palladium-on-carbon catalyst (0 5 g) in ethyl acetate (300 ml) were shaken in a hydrogen atmosphere at room temperature and atmospheric pressure Hydrogen (585 ml) was absorbed during 45—50 min rapidly at the beginning The catalyst was removed by centrifugation and the solution was evaporated to dryness in a rotary evaporator at 30° and reduced pressure The crystalline residue weighed 1 5 g (92 per cent) and melted at 142—148° The colourless needles melted after recrystallization from ethyl acetate at 158—160° $[\alpha]_D^{22} = +6\ 3°$ (C 0 65, methanol) (Found C 59 8, H 5 3, N 12 7 $C_{11}H_{12}N_2O_3$ requires C 60 0, H 5 5, N 12 7)

UV spectrum, abs ethanol λ_{max}=294 nm, log ε=3 70, $\lambda_{shoulder,\ unsharp}$= 235 nm log ε=3 53

IR spectrum (KBr) 3340 and 3410 cm 1 (amine NH_2), 3350 cm 1 (amide NH) 1760 cm 1 (γ-lactone CO), 1650 cm 1 (amide CO)

(S)-(+)-4-(2 benzimidazolyl)-γ-butyrolactone (VI)

Compound (V) (0 20 g) was refluxed for 45 min in a solution of TCA (0 3 g) in carbon tetrachloride (140 ml) in a nitrogen atmosphere The liquid was evaporated at reduced pressure, the residue then dissolved in chloroform (25 ml) and the TCA was removed by extraction with phosphate buffer (1 M, pH 6, 5 ml) The chloroform was dried over anhydrous sodium sulphate and evaporated at

of the R configuration suggests a certain *inversion* during the synthesis Similar results have been noted in the deamination of γ aminovaleric acid (Moll 1966)

As support for this idea it may be mentioned that the specific rotation after repeated recrystallization of compound (II) was greater than the value reported for this compound in the literature (Červinka & Hub 1968) A calculation based on this difference indicates that the ratio between S- and R configuration apparently was of the same order of magnitude for the compound (II) mentioned in the literature (*loc cit*) as for the compound obtained by deamination in the present work

(S)-4-carboxy-γ-butyrolactone (II)

The starting material was (S) (+) glutamic acid (I) (min 99 per cent L (+) glutamic acid as stated by the manufacturer, E Merck) $[\alpha]_D^{25} = +30\ 9°$ (C 1 0, hydrochloric acid, 6 M) Compound (I) was deaminated according to the standard procedure (Plieninger *et al* 1961)

A portion of compound (II) was dissolved in warm diethyl ether After 10—12 h at —20° crystals appeared, that were recovered by filtration After three re crystallizations from diethyl ether, the m p of the crystals was 71—72° $[\alpha]_D^{22} = -4\ 0°$ (C 5 1, water), $[\alpha]_D^{22} = +14\ 2°$ (C 5 6, methanol)

CD spectrum $\lambda_{max} = 214$ nm, $\Delta\varepsilon = +1\ 56$

Lit (Červinka & Hub 1968) m p 71—72°, $[\alpha]_D^{22} = -2\ 8°$ (C 5 8 water), $[\alpha]_D^{22} = +10\ 6°$ (C 5 0, methanol)

The ORD curve exhibits a positive Cotton effect and an intersection point with the axis of zero rotation at 219 nm

(S)-(+)-4-chloroformyl-γ-butyrolactone (III)

The distillate from compound (II) was used without recrystallization in the preparation of compound (III) according to the method described by Plieninger *et al* (1961), and thus it was optically impure

$[\alpha]_D^{22} = +45\ 0°$ (C 0 5, benzene)

CD spectrum (cyclohexane) $\lambda_{max} = 250$, 188 nm, $\Delta\varepsilon = +0\ 13$, $+0\ 30$

(S)-(+)-N-(2-nitrophenyl)-4-carbamoyl-γ-butyrolactone (IV)

2 nitroaniline (1 4 g) and compound (III) (1 5 g) were heated in benzene (5 ml) until no more hydrochloric acid was developed (about 20—30 min), after which an additional quantity of benzene (5 ml) was added A yellow precipitate separated on cooling The crystals were recovered by filtration and washed with a small amount of benzene Yield 2 2 g (87 per cent) Following concentration of the filtrate, an additional quantity of the substance (0 2 g) was obtained from the mother liquor, bringing the overall yield to 95 per cent The product consisted of crystals of two shapes, *viz*, bright yellow needles and yellow prisms It melted at 110—120° $[\alpha]_D^{22} = +49\ 1°$ (C 0 5, chloroform)

Kofler micro hot stage preheated to 185° $[\alpha]_D^{22}=-31.4°$ (C 0 50, hydro chloric acid 1 M), $[\alpha]_D^{22}=-14.8°$ (C 1 0, ammonium hydrogen carbonate, 0 1 M) (Found C 60 0, H 5 6, N 12 5 $C_{11}H_{12}N_2O_3$ requires C 60 0, H 5 5, N 12 7)

UV spectrum, abs ethanol $\lambda_{max}=281$, 274 5 and 244 5 nm, log $\varepsilon=3.83$, 3 81 and 3 72 respectively The UV spectra in different solvents are identical with those of metabolite A (cf Table 14)

The IR spectrum (KBr) illustrated in Fig 36 is identical in every detail with the spectrum of metabolite A shown in Fig 33

The mass spectrum, likewise is identical with the spectrum of metabolite A The spectrum of the metabolite is shown in Fig 34 and that of synthetic product in Fig 44

NMR spectrum Compound (VII) (70 mg) was dissolved in DMSO d_6 (0 5 ml) and the spectrum run at 60 MHz The spectrum displayed a multiplet in the region $\delta=1.8$—2 5 (4H $CH_2 CH_2$) a broadened triplet at $\delta\approx4.85$ (1H, >CH O) an almost symmetrical multiplet centred at $\delta=7.32$ (AA BB spec trum 4H aromatic protons) and a very broad singlet at $\delta=8.13$ (3H, >N H O H CO-O H) The position and shape of the last signal had the same features as described for the NMR spectrum of metabolite A The spectrum was identical with that of metabolite A (cf Fig 35)

SYNTHESIS OF COMPOUNDS FOR NMR AND CD INVESTIGATIONS

(The compounds investigated by means of CD measurements are shown in Table 16 p 97)

(±) 2 (1 hydroxyethyl)benzimidazole

The compound was prepared from o phenylenediamine and (±) lactic acid by refluxing in hydrochloric acid (4 M) according to the standard procedure (Phillips 1928)

NMR spectrum The compound (70 mg) was dissolved in DMSO d_6 (0 5 ml) and the spectrum run at 60 MHz The spectrum displayed a doublet at $\delta=1.53$ J=6 5 cps (3H CH_3) a quartet at $\delta=4.98$ J=6 5 cps (1H >CH O-), a symmetrical multiplet centred at $\delta=7.32$ (AA BB spectrum, 4H aromatic pro tons) and a broad hump overlapping the aromatic multiplet at $\delta=5$—8 8 (2H, >N H and O H) On addition of a drop of D_2O the broad hump disappears and a peak (DHO) appears at $\delta\approx4.4$

(R) (+) and (S)-(—) 2 (1 hydroxyethyl)benzimidazole

The compound were prepared according to the standard procedure (Phillips 1928) starting from R (—) lactic acid (30 per cent aqueous solution containing approx 98 per cent D and 2 per cent L enantiomers according to the manufacturer Sigma Chemical Co) and (S) (+) lactic acid (30 per cent aqueous solution of L (+) lactic acid Sigma Chemical Co)

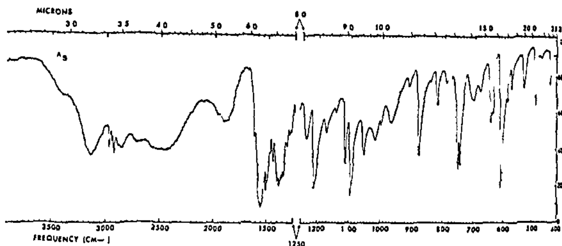


Fig 36 — IR spectrum of synthetic (*S*) (—) 4 (2 benzimidazolyl) 4 hydroxybutyric acid in potassium bromide disk

reduced pressure The residue was dissolved in hot benzene, from which colourless needles separated on cooling Yield 0 17 g (91 per cent) M p 186—188° $[\alpha]^{22}_{D}=+14\ 4°$ (C 0 25, chloroform) (Found C 65 2, H 5 5 N 14 0 $C_{11}H_{10}N_2O_2$ requires C 65 3, H 5 0, N 13 9)

UV spectrum, abs ethanol $\lambda_{max}=281\ 5$, 275, 248 nm, log $\varepsilon=3\ 80$, 3 88 3 81

IR spectrum (KBr) 1780 cm $^{-1}$ (γ lactone CO)

NMR spectrum Compound (VI) (70 mg) was dissolved in DMSO d_6 (about 0 5 ml) and the spectrum run at 60 MHz The spectrum displayed two peaks for the methylene groups at $\delta=2\ 64$ and $\delta=2\ 71$ (4H $CH_2\ CH_2$) a multiplet at $\delta=5\ 80$ (1H, >CH O) and a symmetrical multiplet (AA BB spectrum) at $\delta=7\ 42$, overlapping with a very broad singlet (5H four aromatic protons and one >N-H)

The mass spectrum of the compound will be discussed in Chapter 10

(S)-(—)-4-(2-benzimidazolyl)-4-hydroxybutyric acid (VII)

Compound (V) (0 5 g) was refluxed for 1 h in hydrochloric acid (1 M 4 ml) in a nitrogen atmosphere The solution was concentrated to about 2 ml in a rotary evaporator at reduced pressure and 30° Ammonium hydroxide solution (2 M, 2—3 ml) was added dropwise to pH 6 and the solution was concentrated to 2 ml in a rotary evaporator as mentioned above A precipitate of colourless prisms was obtained, which was recovered by filtration Yield 0 3 g (65 per cent) Additional crystals appeared in the mother liquor following concentration in a rotary evaporator and standing at 0° during 10—12 h The crystals were collected by filtration The weight of this product which also contained a small amount of compound (VI), was 46 mg after washing with chloroform The overall yield was 74 per cent The compound melted at 205—210° (decomp) on a

removed by evaporation in a rotary evaporator at the ambient temperature. This material was placed on top of the column and chromatographed with the developing solvent (ethyl acetate methanol, 4 1, by vol) The separation of the mixture was checked by examination in UV light (254 nm) *The eluates were* investigated spectrophotometrically Fractions containing the benzimidazole derivative were pooled and evaporated to dryness in a rotary evaporator This procedure had to be repeated since the separation from contaminants was not satisfactory after one run The crystalline product obtained weighed 0 15 g, making the overall yield 47 per cent The product melted at 188—198°

After recrystallization *from water containing* 15 per cent ethanol, treating the solution with charcoal and recrystallizing the product from a mixture of ethanol *n-butanol (1 1, by vol)*, colourless needles were obtained M p 194—198° (decomp) $[\alpha]_D^{25}=-41.6°$ (C 0 50, hydrochloric acid, 1 M) (Found C 60 8, H 5 9, N 15 4. $C_9H_{10}N_2O_2$ requires C 60 7, H 5 7, N 15 7)

UV spectrum, methanol $\lambda_{max}=281, 274$ 244 nm, log $\varepsilon=3.87, 3.87, 3.78$

CD spectrum $\lambda_{max}=240$—247 nm, $\Delta\varepsilon\approx-1.75$ Fig 37 (b) shows the CD curve

Lit (Rosenfeld *et al* 1951) for the D form (corresponding to the S-configuration) m p 184—186° (decomp), $[\alpha]_D=+39.6°$ (C 3 6, hydrochloric acid 1 M)

(R)-(—) 2 (1 hydroxybenzyl)benzimidazole (D-(—) HBB)

The compound was synthesized according to Phillips (1928) and Kadin *et al* (1964) with minor modifications

(*R*) (—) mandelic acid (2 3 g) (corresponding to the D form, E Merck) and *o* phenylenediamine (1 1 g) were refluxed for 3 h in hydrochloric acid (4 M, 15 ml) in a nitrogen atmosphere On cooling, the solution was neutralized drop wise with ammonium hydroxide solution (25 per cent) The mixture was *extracted* twice with methylene chloride (25 ml) The pooled organic phase was washed with sodium hydrogen carbonate (1 M, 20 ml), dried over anhydrous sodium sulphate and evaporated in a rotary evaporator at ambient temperature The residue was extracted with warm (80°) water (200—250 ml) and treated with charcoal. *Colourless needles* separated on cooling and were collected by filtration The yield was 0 9 g (43 per cent based on *o* phenylenediamine), m p 94—96° $[\alpha]_D^{25}=-92.2°$ *(C 0 50 hydrochloric acid 1 M)*, $[M]_D^{25}=-221.2°$ (Found C 73 0, H 5 8 N 12 0 $C_{14}H_{12}N_2O$ 1/3 H_2O requires C 73 0, H 5 5, *N 12 1*) After drying *in vacuo* for 3 h at 85° the compound melted at 81—84° *(Found* C 75 0, H 5 3 N 12 4 $C_{14}H_{12}N_2O$ requires C 75 0, H 5 4, N 12 5)

CD spectrum $\lambda_{max}\approx282$ 273, 245, 220 nm, $\Delta\varepsilon=-2.44, -2.02, +7.30, -21.2$

Lit (Kadin *et al* 1964) for the hydrochloride of the D form *(corresponding to* the R-configuration), $[\alpha]_D^{23}=-82.7°$ *(C 1, water)*, $[M]_D^{25}=-216.6°$

(R) (+) 2 (1 hydroxyethyl)benzimidazole

M p 179—180° $[\alpha]_D^{25} = +32\ 0°$ (C 4 0, ethanol, abs)

UV spectrum methanol λ_{max}=281, 274, 244 nm, log ε=3 87, 3 87, 3 79

CD spectrum λ_{max}=241 nm, Δ ε=+1 10

(S) (—) 2 (1 hydroxyethyl)benzimidazole

M p 181—182° $[\alpha]_D^{25} = -33\ 8°$ (C 4 0, ethanol, abs)

CD spectrum λ_{max}=243—247 nm, Δ ε=—1 00 Fig 37 (a) shows the CD curve

Lit (Dimler & Link 1942) for the D and L forms, (corresponding to the *R* and *S* configurations), m p 175—177°, $[\alpha]_D = \pm 33\ 4°$ (C 4, ethanol)

(R)-(—)-2 (1,2 dihydroxyethyl)benzimidazole

The compound was prepared according to the procedure of Fedoroňko *et al* (1968), with minor modifications

(*S*) (—) glyceraldehyde[17] (0 46 g) was dissolved in water (20 ml) saturated with nitrogen and added to a solution of *o* phenylenediamine (0 48 g) dissolved in water (20 ml) saturated with nitrogen, the two solutions being mixed in a flask filled with nitrogen The solution was kept in darkness at room temperature during a week and was subsequently evaporated in a rotary evaporator at room temperature The yellow residue was treated with acetone several times and the slightly coloured precipitate was collected by filtration The yield was 0 22 g (28 per cent) The crude product melted at 187—189° (decomp) The acetone solutions were recombined and evaporated at reduced pressure It was possible to recover an additional quantity of the product by subjecting the residue to DCC

Purification of the crude acetone extract by means of DCC As the compound separated well in TLC using silica gel GF_{254} and with ethyl acetate methanol (4 1, by vol) as the developing solvent mixture (R_f=0 40) it was decided to use DCC (*cf* Loev *et al* 1965, 1967) to separate and isolate the compound from the impurities present in the mother liquor of the crude product

A mixture of ethyl acetate (7 ml) and methanol (7 ml) was added to silica gel (125 g) prepared for dry column chromatography (M Woelm) in a glass stoppered flask under vigorous agitation The flask was then left for 10—12 h to allow the contents to equilibrate A cellulose dialysis bag (37 mm wide and 60 cm long) was prepared (*cf* Asmundson *et al* 1969) One end of this tubing was tied by a knot and constituted the bottom of the column Some glass wool (extra fine grade p 10) was first placed in the tubing which was then packed with the equilibrated silica gel The remaining part of the acetone extract (*cf* above) was dissolved in a few ml of methanol Silica gel (5 g) was added, and the methanol was then

[17] The composition of the chemical used was as follows 90 per cent L (—) glyceraldehyde+ 10 per cent water $[\alpha]_D = -8\ 7 \pm 0\ 5°$ (C 2 water) according to the manufacturer (Fluka AG Buchs S G Switzerland)

Table 15 — UV spectra of benzimidazole and some 2 substituted benzimidazole derivatives in absolute ethanol

[a] Rabiger & Joullié (1964)
[b] Raines & Kovacs (1967), methanol
[c] Leandri et al (1955)

Compound	λ_{max}nm (log ε)							
benzimidazole[a]	279	(3 81)	272	(3 82)	243	(3 68)		
2 ethylbenzimidazole[a]	280	(3 89)	272	(3 91)	243	(3 80)		
2 propenylbenzimidazole[b]	295	—	—	—	—	—		
2 acetylbenzim dazole[a]	300	(3 92)	—	—	235	(3 65)		
2 (2 furyl)benzimidazole[c]	321	(4 36)	307	(4 45)	250	(4 08)	245	(4 05)
metabolite A	281	(3 83)	274	(3 81)	244 5	(3 72)		

such as >C=O, CH=CH and [ring](X=O, S), *i e*, groups that form conjugated double bonds with benzimidazole, leads to a shift in the absorption maxima at 279 and 272 nm to higher wavelengths (*cf* Table 15) (*cf* Rabiger & Joullie 1964 Leandri *et al* 1955) accompanied by a change in the fine structure of the UV spectrum of the benzimidazole These data, compared with the observations made in the present work as mentioned above, suggest a conversion in the 2-substituent of the starting material, the furan nucleus, caused by metabolic action, and leading to the disappearance of the double bond system conjugated with the benzimidazole moiety

The character of the change was suggested to some extent by the IR spectrum of metabolite A, which had absorption bands at 2960, 2940 and 2920 cm^{-1} arising from C H stretching vibrations, indicating the presence of aliphatic groups in the molecule The occurrence of such groups was substantiated by the NMR spectrum (Fig 35) which showed a four proton signal at δ= 1 7—2 7 that was attributed to two methylene groups, and a one proton signal at δ=4 83 that was associated with a methine proton The chemical shift of this proton indicates a strong deshielding effect which is probably caused by a free or esterified hydroxyl group The NMR spectrum further shows that the benzene nucleus in the benzimidazole moiety is unsubstituted since the four proton signal in the aromatic region at δ=7 26 features a symmetric AA BB pattern, which is caused by the four protons 4/7 and 5/6 (Black & Heffernan 1962)

For these reasons it was assumed that the furan nucleus had become transformed in the course of metabolism into an open carbon chain comprising two methylene groups one methine group containing an alcoholic hydroxyl, and a terminal carboxyl *i e*, a substance with the structure 4 (2 benzimidazolyl) x hydroxybutyric acid which would have the composition $C_{11}H_{12}N_2O_3$ This view is supported by the presence of a strong absorption band at 1560 cm^{-1}, in fact the strongest band in the spectrum This band has been attributed to a carboxylate group (*cf* Bellamy

(S)-(+)-2-(1-hydroxybenzyl)benzimidazole (L-(+)-HBB)

(*S*)-(+)mandelic acid (1 2 g) (corresponding to the L form, Fluka AG) and *o* phenylenediamine (5 0 g) were refluxed for 3 h in hydrochloric acid (4 M, 8 ml) in a nitrogen atmosphere and treated as described for the *R* enantiomer IR spectroscopy revealed the presence of impurities in the crude crystalline material The compound was hence purified by column chromatography on silicic acid (100 mesh) (Mallinckrodt, St Louis, USA)

A glass column (15 mm i d) was packed with silica gel (5 g) in chloroform The crude product (0 1 g) was dissolved in chloroform (1 ml), introduced at the top of the column and eluted with the same solvent The purified product was detected by UV investigation of the eluates, and the fractions containing the benzimidazole derivative were collected The pooled fractions were evaporated in a rotary evaporator at reduced pressure and at room temperature The residue was dissolved in warm (80°) water Needle-shaped crystals separated on cooling The compound was collected by filtration, and the yield was 65 mg (49 per cent based on *o* phenylenediamine) The compound was dissolved at 70° in water containing 15 per cent ethanol, yielding colourless needles on cooling, m p 94—96° $[\alpha]_D^{25}$ = +95 8° (C 0 50, hydrochloric acid, 1 M), $[M]_D^{25}$ = +220 2° (Found C 73 0, H 5 6, N 11 8 $C_{14}H_{12}N_2O$ 1/3 H_2O requires C 73 0, H 5 5, N 12 1) The product was re analyzed after drying *in vacuo* for 3 h at 85° (Found C 75 1, H 5 3, N 12 5 $C_{14}H_{12}N_2O$ requires C 75 0, H 5 4, N 12 5)

UV spectrum, methanol λ_{max}=282,275, 246 nm, log ε=3 95, 3 97, 3 84

CD spectrum λ_{max}=281 275, 245, 219 nm, Δ ε= +2 34 +1 84, —7 13, +21 6 Fig 37 (c) shows the CD curve of the compound

Lit (Kadin *et al* 1964) for the hydrochloride of the L form (corresponding to the *S* configuration), $[\alpha]_D^{25}$ = +83 2° (C 1, water), $[M]_D^{25}$= +216 9°

DISCUSSION

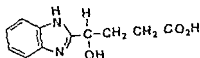
I STRUCTURAL ELUCIDATION OF METABOLITE A

On dissolution in water the concentrate of metabolite A from horse urine (Chapter 6) gave a precipitate which, following recrystallization from water, yielded a light brownish substance Chromatography on a Sephadex column as described in Chapter 3 resulted in isolation of metabolite A which appeared to be a colourless crystalline substance The compound, which was optically active and levorotatory, had the molecular formula $C_{11}H_{12}N_2O_3$, mol wt 220 and melted at 206—210°

The UV spectrum of this compound (Figs 12 and 37 (f)) differed considerably from that of the starting material, FB (Fig 2) but bore a close resemblance to the UV spectra of benzimidazole and 2 alkyl substituted benzimidazoles (*cf* Table 15) Thus, there were two absorption maxima at 281 and 274 nm and one maximum at 245 nm The corresponding absorption maxima of benzimidazole are at 279, 272 and 243 nm. 2 Substitution of the benzimidazole moiety with groups

ole moiety implying that this carbon is the hydroxyl bearing secondary carbon atom. The secondary carbon is thus C 4 of the butyric acid

The optical activity $[\alpha]_D^{22}$ = —31 5°, confirms the presence of an asymmetric centre in the molecule According to the proposed structure of *metabolite A the C-4 carbon of the butyric acid* must be the asymmetric centre This acid may be *regarded* as a benzimidazole derivative of a substituted lactic acid or that of 2 hydroxyglutaric acid For benzimidazole derivatives of aldonic acids (polyhydroxy acids) the benzimidazole rule (Richtmyer & Hudson 1942) states Whenever the hydroxyl group on the second atom of an aldonic acid is on the right in the conventional projection formula of Fischer the rotation of the derived benzimidazole is positive and conversely when the hydroxyl group is on the left, the rotation of the benzimidazole is negative Hence if the structural formula of metabolite A is drawn with the carbon chain vertical and the benzimidazole on top then the hydroxyl on the carbon atom adjacent to the benzimidazole should be pla[illegible] otation in accordance with the [illegible] that metabolite A is (S) (—)-[illegible] the structural formula



To prove the correctness of this structure the compound concerned has been prepared synthetically A comparison of the UV IR, mass and NMR spectra specific rotation and other data proved identity between metabolite A and the synthetic product

Metabolite A as isolated from dog urine was identical in every respect with metabolite A from horse urine

II ABSOLUTE CONFIGURATION OF METABOLITE A

The way of synthes s illustrated in Diagram 3 (p 85) was chosen since it could be expected to proceed with retention of the configuration of the starting material Th s could be concluded from investigations of the deamination of (S) (+) glutamic acid to yield (S)-4-carboxy-γ-butyrolactone (compound II in Diagram 3) with retention of the configuration (Austin & Howard 1961 Cervinka & Hub 1968) Considering the other steps in Diagram 3 to be harmless with respect to the risk of inversion and applying the "benzimidazole rule (*Richtmyer & Hudson* 1942) to metabolite A (S) (+) glutamic acid was chosen as the *starting* material.

The stereochemical invest gation of metabol te A and the corresponding synthetic product was effected by means of *circular dichroism* (CD) measurements The CD of a compound depends on the *difference in* the absorption coefficient for left and r ght handed circularly polarized light as the wavelength changes CD *maxima*

1968) Morgan (1961) observed that the carbonyl band was replaced by a broad band at 1550 cm 1 in the IR spectrum of 3 (2 benzimidazolyl)propionic acid and regarded it as a zwitterionic compound The anodic migration in paper electro phoresis above pH 4 (Table 13) is further indicative of a carboxyl group

The structure suggested is also corroborated by other observations of metabolite A in its NMR and IR spectra The NMR spectrum shows a singlet of a three proton signal at δ=9 07 This signal is attributed to the protons from >NH, OH and COOH and results from the fast acid catalyzed proton exchange intermolecularly and/or intramolecularly and *via* the solvent (DMSO) This observation and the strong absorption bands in the region 3600—1700 cm 1 of the IR spectrum indicating strong intermolecular and/or intramolecular hydrogen bonds, are in agreement with what can be expected from a compound with the structure suggested above

In the previous discussion in this chapter the position of the secondary carbon atom remained an open question As mentioned, the signal at δ=4 83 was at tributed to the proton of the >CH O group The chemical shift of this proton however, is explained only to a certain extent by the attachment of an alcoholic hydroxyl group to the methine group The further deshielding of the methine proton is assumed to be caused by the benzimidazole moiety

The chemical shift of the proton in the >CH O group attached to the 2 benzi midazolyl group was checked by NMR spectroscopy of 2 (1 hydroxyethyl)benzi midazole The spectrum shows the single proton resonance at δ=4 98 a value very close to that of the methine proton of metabolite A This would indicate that the methine group of the metabolite is directly attached to the C 2 carbon of the benzimidazole implying that it is probably the C-4 carbon of the butyric acid

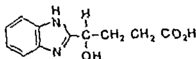
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In cases of direct attachment of an alkyl substituent to C 2 of the benzimidazole moiety the fragmentation pattern is different (Nishiwaki 1968)

Since all features typical of mass spectra of 2 benzimidazolylcarbinols are also present in the mass spectrum of metabolite A it seems quite plausible that the carbon atom of the methine group is attached directly to the C 2 of the benzimida

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To prove the correctness of this structure the compound concerned has been prepared synthetically A comparison of the UV, IR, mass, and NMR spectra, specific rotation and other data proved identity between metabolite A and the synthetic product.

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Since all features typical of mass spectra of 2 benzimidazolylcarbinols are also present in the mass spectrum of metabolite A, it seems quite plausible that the carbon atom of the methine group is attached directly to the C 2 of the benzimida

Table 16 — Name of starting materials Structural formula and absolute configuration of corresponding benzimidazole derivatives investigated Signs and values of differential absorption ($\Delta\varepsilon$) in the neighbourhood of 245 nm. (BI=2 benzimidazolyl)

Starting material	Structural formula & absolute configuration of benzimidazole derivative		$\Delta\varepsilon$ λ_{max}=245 nm
(*R*) (—) lactic acid	BI—C(OH above, H below)—CH_3	(*R*)	+1 10
(*R*) (—) mandelic acid	BI—C(OH above, H below)—C_6H_5	(*R*)	+7 30
(*S*) (+) lactic acid	BI—C(H above, OH below)—CH_3	(*S*)	—1 00
(*S*) (—) glyceraldehyde	BI—C(H above, OH below)—CH_2OH	(*R*)	—1 75
(*S*) (+) mandelic acid	BI—C(H above, OH below)—C_6H_5	(*S*)	—7 13
(*S*) (—) 4 (2 benzimidazolyl) 4-hydroxybutyric acid (synthetic)	BI—C(H above, OH below)—CH_2 CH_2 CO_2H	(*S*)	—1 45
Metabolite A			—1 52

occur only in the vicinity of absorption bands (*cf* Crabbe 1965, Klyne & Scopes 1969) Figure 37 (d) and (e) illustrate CD curves of the synthetic product and metabolite A respectively The UV curve of the metabolite is given in (f) The CD curves show maxima, positive in the 280 nm and negative in the 245 nm region As could be expected the CD maxima at these wavelengths coincide with the UV maxima of these compounds The structural identity of metabolite A and the synthetic product has already been established by IR, NMR, mass spectroscopic and other investigations The CD investigations, demonstrating the identity

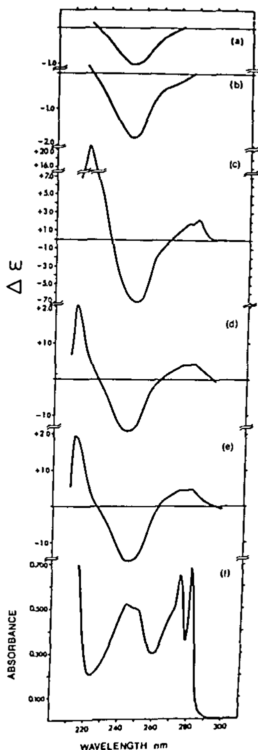
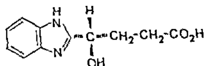


Fig 37 — Circular dichroism (CD) spectra in methanol of benzimidazole derivatives of (a) (*S*) (+) 'actic acid, (b) (*S*) (—) glyceraldehyde and (c) (*S*) (+) mandelic acid CD spectra in metharol of (d) synthetic (*S*) (—) 4 (2 benzimidazolyl) 4-hydroxybutyric acid, and (e) metabolit A (f) shows the UV spectrum of metabolite A (1×10^{-4} M in abs ethanol)

close to 245 nm These results are in agreement with ORD investigations of structurally related benzimidazole derivatives, previously reported in the literature

Consequently, the asymmetric carbon in metabolite A has the *S*-configuration and the compound has in regard to the absolute configuration the structural formula as shown below



III ON THE FORMATION OF METABOLITE A IN MAMMALIAN METABOLISM

The mammalian metabolism of aromatic and heterocyclic compounds by hydroxylation has been thoroughly investigated during a long period of time Comprehensive reviews on this topic have been published by Williams (1959), Gerarde (1960), Browning (1965) and others For this reason it has been assumed, that furan as well being a heterocyclic compound, would undergo hydroxylation in the course of animal metabolism (Williams 1959, *f*) Only recently, Trudgill (1969) demonstrated that 2 furoic acid is subjected to hydroxylation in the course of a microbial metabolism. A search of the literature has not revealed any information concerning the fate of furan or 2 substituted furan derivatives in mammalian metabolism

It has been shown that arene oxides are formed in the microsomal oxidation of some aromatic compounds (Jerina *et al* 1969, *cf* also Chapter 5) It has also been suggested that arene oxides play a major role in the metabolism of aromatic compounds leading to the formation of phenols, dihydrodiols and premercapturic acids The formation of phenols may occur by means of a cationoid intermediate (Daly *et al* 1968 Jerina *et al* 1969)

The structural elucidation of metabolite A has demonstrated that the furan nucleus of FB undergoes a cleavage and conversion into an open aliphatic chain during the metabolism of FB

It may be assumed that the initial stage in the sequence leading to the cleavage of the furan nucleus is an enzymatic hydroxylation If this hydroxylation is microsomal which seems probable, and if it occurs in analogy with the process suggested for the microsomal oxidation of aromatic substances, the cationoid intermediate (1), as shown in Diagram 4, would be stabilized as 4-(2 benzimidazolyl) 3-buten-4-olide (2) The continued metabolism of such a compound would probably comprise a hydrolysis, leading to the formation of 4-(2 benzimidazolyl)-4-oxo-butyric acid (3) which would subsequently be reduced to (*S*) (—) 4 (2 benzimidazolyl)-4 hydroxybutyric acid (4)

The result obtained by studies of the microbial metabolism of 2 furoic acid (Trudgill 1969) may be mentioned here as support of the proposed scheme A

of the curves for the two compounds, clearly show that they are identical from a stereochemical point of view as well This together with the conditions of the synthesis indicate the *S* configuration in both

This view was further corroborated when the CD curve of metabolite A was compared with CD curves of structurally related benzimidazole derivatives that had been synthesized from compounds with known absolute configurations (a) (b) and (c) in Fig 37 illustrate CD curves of benzimidazole derivatives of (*S*) (+) lactic acid, (*S*) (—) glyceraldehyde and (*S*) (+) mandelic acid In all of these CD curves the CD maximum in the vicinity of 245 nm is negative

The structural formulae of these benzimidazole derivatives are also stereochemically closely related and are illustrated in Table 16 Thus, the hydroxyl group attached to the chiral carbon atom is in these compounds on the left in the projection formula of Fischer (see the text on p 95) Consequently there are very good reasons to believe that metabolite A as well, showing a negative CD maximum in the neighbourhood of 245 nm has the same chirality at the asymmetric centre and possesses the *S* configuration Benzimidazole derivatives of (*R*) (—) lactic acid and (*R*) (—) mandelic acid have also been prepared synthetically CD curves of these products displayed, as expected a mirror image relationship to those of the *S* enantiomers *i a*, positive CD maxima of the same magnitude of $\Delta \varepsilon$ in the vicinity of 245 nm (*cf* Table 16)

The results of this investigation are in agreement with the investigations reported by other authors, on *optical rotatory dispersion* (ORD) of benzimidazole derivatives structurally related to the compounds investigated by CD in the present work Thus, Chilton & Krahn (1967, 1968) reported on ORD investigations of polyhydroxy benzimidazole derivatives (benzimidazole derivatives of aldonic acids) It can be seen from these studies that the configuration at the first carbon atom attached to the C-2 of the benzimidazole moiety determines the sign of the Cotton effect in the vicinity of 245 nm The relationship between the sign and the absolute configuration coincides with the findings of the present investigation

As noted above the benzimidazole derivative of (*R*) (—) mandelic acid has a positive CD maximum at 245 nm (*cf* Table 16) It is in complete agreement with this finding that an ORD investigation on the benzimidazole derivative of (*R*) (—) mandelic acid has demonstrated a positive Cotton effect of the substance in the vicinity of 245 nm (Neilson 1967)

The results of the present stereochemical investigations may be summarized as follows A comparison of CD curves of synthetic (*S*) (—) 1 (2 benzimidazolyl) 4 hydroxybutyric acid and metabolite A shows that both compounds are characterized by a negative CD maximum in the vicinity of 245 nm indicating the same configuration in the two compounds, probably the *S*-configuration as the synthesis was carried out with retention of the configuration

CD curves of benzimidazole derivatives of (*S*) (+) lactic acid (*S*) (—) glyceraldehyde and (*S*) (+) mandelic acid all have a negative CD maximum

chain of reactions was postulated according to which the furan nucleus of the primarily formed 2 furoyl CoA is hydroxylated, leading to the formation of 5-hydroxy 2 furoyl CoA which, being a furanol, undergoes enol keto tautomerism and is rearranged to a 3 buten-4 *olide*, (5-*oxo* Δ^2 dihydro 2 furoyl CoA) (Trudgill 1969) Finally, 2-oxo glutaric acid *is formed* by enzymatic hydrolysis as the last link in the reaction chain Diagram 4 also includes the furan hydroxylation as suggested by Trudgill

In the hydroxylation of 2 *furoic* acid by enzymes of bacterial origin the oxygen is derived from water (Kitcher & Trudgill 1970) whereas molecular oxygen is [illegible] the m crosomal oxidation (*cf* Holtzman *et al* 1967) It is not possible, [illegible]

[illegible]
evidence is available

The last step in the scheme finds support in the enzymatic reduction of keto groups to alcohol groups, *e g* *the reduction of* 2-oxo-glutaric acid *Such* enzymatic reductions have been observed in a number of biological systems *Of greater interest in this connection is the metabolism of* 4 aryl-4 oxo-butyric acids whose biological conversion results in products showing structural similarities *to metabolite A Thus* (—)-4 hydroxy-4-phenylbutyric acid has been isolated from various mammals such as the dog in the metabolism of 4 oxo-4 phenylbutyric acid, a *process associated with simultaneous* excretion of small amounts of the dextrorotatory acid (Thierfelder & Schempp 1917 1921) An extensive survey of the literature concerning this type of metabolism has been reported *by* Bowman (1968) who in the 1960s carried out investigations of the degradation of nicotine in mammals in collaboration with McKennis and co workers (for references, *cf* Bowman 1968) This team observed that 4-oxo-4 (3 pyridyl)butyric acid is an important link in one of the degradation pathways In the dog this compound is metabolized to (—)-4 hydroxy-4 (3 pyridyl)butyric acid It was assumed that the dextrorotatory form may have occurred as well, but in smaller quantities (McKennis *et al* 1964)

It is a notable feature that all three structurally related metabolites are optically active and in fact levorotatory As a result of ORD investigations on homologues of (*R*) (—) mandelic acid the *S* configuration has been assigned to (—) 4 hydroxy 4 phenylbutyric acid (Randinitis *et al* 1970) Consequently, the *S* configuration may also be attributed to the structurally identical levorotatory metabolite of 4 oxo-4 phenylbutyric acid (Thierfelder & Schempp 1917, 1921)

Also the (—) 4 hydroxy-4 (3 pyridyl)butyric acid isolated from dog urine (Mc Kennis *et al* 1964) probably has the *S*-configuration, to judge from the absolute configuration of *R* (+) methyl 3 pyridylmethanol (Červinka *et al* 1970)

Metabolite A also has the *S* configuration quite in agreement with the configuration of the two structurally analogous metabolites It would seem probable under these conditions that the precursor of metabolite A is 4 (2 benzimidazolyl) 4-oxo-butyric acid and that the same enzymatic system is used for reducing the

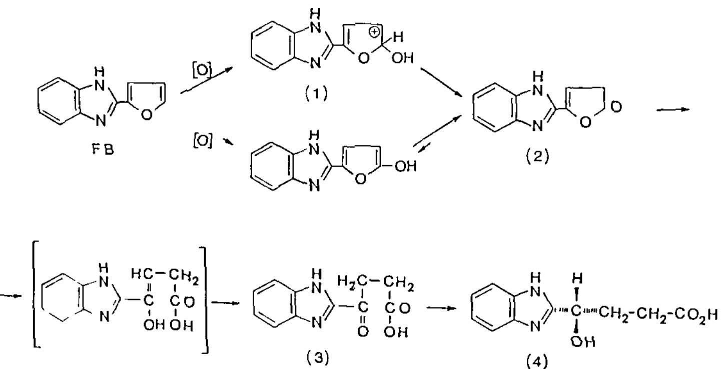


Diagram 4 —Proposed pathway in the formation of (*S*) (—) 4 (2 benzimidazolyl) 4 hydroxy butyric acid (metabolite A) by cleavage of the furan nucleus in the mammalian metabolism of 2 (2 furyl)benzimidazole (FB)

chain of reactions was postulated according to which the furan nucleus of the primarily formed 2 furoyl CoA is hydroxylated leading to the formation of 5 hydroxy 2 furoyl CoA which, being a furanol undergoes enol keto tautomerism and is rearranged to a 3 buten 4 olide (5-oxo Δ^2 dihydro-2 furoyl CoA) (*Trudgill* 1969) Finally 2-oxo glutaric acid is formed by enzymatic hydrolysis as the last link in the reaction chain Diagram 4 also includes the furan hydroxylation as suggested by Trudgill

In the hydroxylation of 2 furoic acid by enzymes of bacterial origin the oxygen is derived from water (Kitcher & Trudgill 1970) whereas molecular oxygen is utilized in the microsomal oxidation (*cf* Holtzman *et al* 1967) It is not possible, however to draw any definite conclusions concerning the mechanism of the hydroxylation of the furan nucleus of FB in the mammalian organism until further evidence is available

The last step in the scheme finds support in the enzymatic reduction of keto groups to alcohol groups *e g* the reduction of 2 oxo glutaric acid Such enzymatic reductions have been observed in a number of biological systems Of greater interest in this connection is the metabolism of 4 aryl 4 oxo-butyric acids whose biological conversion results in products showing structural similarities to metab ol te A. Thus (—) 4 hydroxy 4 phenylbutyric acid has been isolated from various mammals such as the dog in the metabolism of 4 oxo 4 phenylbutyric acid a process associated with simultaneous excretion of small amounts of the dextro rotatory acid (Thierfelder & Schempp 1917 1921) An extensive survey of the literature concerning this type of metabolism has been reported by Bowman (1968) who in the 1960s carried out investigations of the degradation of nicotine in mammals in collaboration with McKennis and co workers (for references *cf* Bowman 1968) This team observed that 4-oxo-4 (3 pyridyl)butyric acid is an important link in one of the degradation pathways In the dog this compound is metabolized to (—) 4 hydroxy 4 (3 pyridyl)butyric acid It was assumed that the dextrorotatory form may have occurred as well but in smaller quantities (McKen nis *et al* 1964)

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three 4 aryl 4 oxo butyric acids with a certain degree of stereospecificity No ob servations have been made that might suggest the formation of the dextrorotatory form as well in the metabolism of FB It is possible, however, that the racemate may have been lost in the procedure of purification If this is the case the racemate must be more easily soluble than the optically active form and the *R* configuration is probably present only in small amounts

It has not yet been possible to prove the existence of any of the intermediate metabolites proposed in the scheme, such as 4 (2 benzimidazolyl) 3 buten 4 olide (2) and 4 (benzimidazolyl) 4 oxo butyric acid (3) (Diagram 4, p 100)

SUMMARY

One of the urinary metabolites of FB has been denoted metabolite A It has been found in the urine of each animal species investigated in the present work, following FB administration The metabolite was isolated from concentrates of this substance from horse and dog urine

The identification as performed by polarimetry, UV, IR, NMR, and mass spectroscopy, and by other means, has proved that metabolite A is (*S*) (—) 4 (2 benzimidazolyl) 4 hydroxybutyric acid This structure was confirmed by syn thesis The metabolites isolated from horse and dog urine have proved to be identical

The absolute configuration has been determined by means of CD measurements of metabolite A, the corresponding synthetic product, as well as the benzimidazole derivatives of (*R*) (—) and (*S*)·(+) lactic acid, (*R*) (—) and (*S*) (+)· mandelic acid, and (*S*) (—)glyceraldehyde The investigation established the absolute configuration of the asymmetric centre in metabolite A as *S*

In the course of this study it was previously shown that the hydroxylation of the benzene nucleus is one of the main pathways in the mammalian metabolism of FB An other important way of degradation is disclosed by the formation of metab olite A, presumably effected by an enzymatic attack on and cleavage of the furan nucleus of FB

Chapter 10. Mass spectrometric investigation of certain substituted alkyl-(2-benzimidazolyl)-carbinol derivatives and 4-(2-benzimidazolyl)-γ-butyrolactone

The mass spectrometric investigation was of primary importance to the structural elucidation of metabolite A (Chapter 9) The spectrum of metabolite A showed a pattern of prominent peaks that have also been found in the spectra of certain (2 *benzimidazolyl*)carbinol[18] derivatives This observation suggested that the isolated metabolite A might be a derivative of such a compound In the present chapter it will be demonstrated that the fragment ions observed are characteristic of the type of substance investigated in this work alkyl (2 benzimidazolyl)carbinols, and independent of variations in the substitution pattern of the alkyl side chain

EXPERIMENTAL

APPARATUS

Low resolution mass spectra were measured on an LKB 9000 mass spectrometer, using the direct inlet system The temperature of the ion source was about 270° and the energy of the ion beam 70 eV Probe temperatures are given in Table 17 The data were plotted by an IBM 1800 computer

High resolution mass spectrometry was performed on an Atlas SM1 instrument (Varian MAT GmbH Bremen Ger) using a resolution of 15 000—20,000 and with perfluorokerosene as reference The ion source temperature was 225° The Ilford photoplates were evaluated in a Gaertner mass spectrometer photoplate comparator (Gaertner Scientific Corp , Chicago USA) All measurements are correct to within 10 ppm

SYNTHESIS OF COMPOUNDS FOR MASS SPECTROMETRIC INVESTIGATIONS

The compounds investigated are listed in Table 17 The syntheses of compounds V VI and VII of which the latter is identical with metabolite A have been described in Chapter 9 Compounds I II III and IV were synthesized as follows

(S) (—) 2-(1 hydroxy-3 methylbutyl)benzimidazole (I)

The compound was prepared in analogy with the standard procedure (Phillips 1928) (S) (—) 2 hydroxyisocaproic acid (0 5 g) (corresponding to the L form, Sigma Chemical Co) and *o* phenylenediamine (0 4 g) were refluxed for 5 h in hydrochloric acid (4 M, 5 *ml*) in a nitrogen atmosphere A white precipitate

[18] In the present chapter the carbinol nomenclature has been adopted

Table 17 — Structural formula and probe temperature for the compounds investigated

General formula: BI-C(1)(OH)-C(2)-C(3)- (BI=2 benzimidazolyl)

Compound	Probe temp	Compound	Probe temp
I BI C(H)(OH) CH CH$(CH_3)_2$	25°	IV BI C(OH)(H)—C(H)(OH) CH OH	120°
II BI C(OH)(H)—C(H)(OH) CO_2H	190°	V BI C(H)(OH) CH OH	25°
III BI C(OH)(H)—C(H)(OH) CO CH_3	25°	VI BI (H, O, O ring)	100°
		VII BI C(H)(OH) CH CH CO_2H	237°

separated on keeping at the ambient temperature The yield was 0 7 g (75 per cent) The colourless prisms obtained by recrystallization from isopropanol melted at 202—206° (Found C 59 9 H 7 0 N 11 4 $C_{12}H_{16}N_2O$ HCl requires C 59 9 H 7 1, N 11 6) Thus the isolated product was the hydrochloride of the synthesized benzimidazole derivative The free base was isolated as follows

The hydrochloride (0 46 g) was dissolved in water (2 ml) and neutralized by adding ammonium hydroxide solution (2 M) dropwise A white precipitate occurred which was collected by filtration and washed with water The product was dissolved in hot methanol (2 ml) Colourless plates separated following the addition of water (0 5 ml) The yield was 0 35 g (89 per cent) m p 174—175° $[\alpha]_D^{25}$ =—35 6° (C 0 50, hydrochloric acid 1 M) (Found C 70 7 H 7 8 N 13 8 $C_{12}H_{16}N_2O$ requires C 70 6, H 7 9, N 13 7)

UV spectrum methanol λ_{max}=281 274 5 241 nm log ε=3 90 3 90 3 80

(2R, 3S) (+)-2,3-dihydroxy-3-(2-benzimidazolyl)propionic acid (II)

(2R,3R) (+) tartaric acid (4 5 g) (corresponding to the L-form; E Merck) and *o* phenylenediamine (2 2 g) were refluxed for 5 h in hydrochloric acid (4 M; 25 ml) in a nitrogen atmosphere The mixture was cooled and treated with sodium hydroxide solution (40 per cent, about 10 ml) until no more precipitate was formed (pH 5) Solid sodium hydrogen carbonate was added to the slurry until no further re dissolution of the precipitate occurred and the solution filtered. The yellow-coloured filtrate was evaporated in a rotary evaporator until a precipitate separated, which was dissolved by adding hydrochloric acid (4 M), until no more carbon dioxide was released The solution, with pH 4, was then concentrated in a rotary evaporator at 30° A yellow precipitate formed on maintaining the solution at 0° for 10—12 h The product was recovered by filtration, and the crystalline mass was then extracted with ethanol, filtered and evaporated in a rotary evaporator Finally, the residue was recrystallized from water (once) and ethanol (96 per cent, twice) to yield colourless needles weighing 2 1 g NMR spectroscopy revealed that the product contained ethanol of crystallization in a ratio of 1 1 The yield was 40 per cent calculated as ethanolate and based on *o*-phenylenediamine Melting behaviour The transparent crystals became opaque at 126—130° (gas evolution), the solid white mass softened at 162° and melted at 165—170° $[\alpha]_D^{25} = +78\,0°$ (C 0 50, hydrochloric acid, 1 M) (The elementary analysis was performed after keeping the compound *in vacuo* during 4 h at 120—125° Found C 53 9, H 4 6, N 12 3 $C_{10}H_{10}N_2O_4$ requires C 54 1, H 4 5; N 12 6)

UV spectrum, methanol $\lambda_{max} = 281$, 276 5, 270 5 and 244 nm, log $\varepsilon = 3\,79$, 3 91, 3 89, and 3 74

IR spectrum (KBr) 1650, 1640 and 1620 cm⁻¹ (carboxylate)

methyl (2R, 3S)-(+)-2,3-dihydroxy-3-(2-benzimidazolyl)propionate (III)

Compound (II) (1 1 g) was refluxed for 7 h in a mixture of methanol (40 ml) and concentrated sulphuric acid (4 ml) After cooling and subsequent neutralization with ammonium hydroxide solution (25 per cent, about 8 ml), the mixture was concentrated to 10 ml at reduced pressure and room temperature It was then treated with ethyl acetate (150 ml) A white precipitate separated, which consisted of inorganic salts The solution was filtered and evaporated in a rotary evaporator The crystalline residue (1 0 g) was dissolved in aqueous methanol (50 per cent) and treated with charcoal White crystals separated when the solution was kept at 0° for 10—12 h The product was recovered by filtration and subsequently recrystallized from aqueous methanol (50 per cent), resulting in colourless plates The yield was 0 8 g (87 per cent) The plates melted at 187—189° (Found C 55 8, H 5 2, N 11 7 $C_{11}H_{12}N_2O_4$ requires C 55 9, H 5 1, N 11 9) $[\alpha]_D^{25} = +77\,9°$ (C 0 50, hydrochloric acid, 1 M)

UV spectrum methanol $\lambda_{max} = 281$, 274, 245 nm, log $\varepsilon = 3\,94$, 3 93, 3 80

IR spectrum (KBr) 1730 cm⁻¹ (ester CO)

Table 17 — Structural formula and probe temperature for the compounds investigated

General formula: 2-benzimidazolyl–C(1)(OH)–C(2)–C(3)– (ring NH, C2 numbered 1, 2, 3 along the side chain) BI=2-benzimidazolyl

Compound		Probe temp	Compound		Probe temp
I	BI–C(H)(OH)–CH–CH(CH_3)(CH_3)	25°	IV	BI–C(OH)(H)–C(H)(OH)–CH OH	120°
II	BI–C(OH)(H)–C(H)(OH)–CO_2H	190°	V	BI–C(H)(OH)–CH OH	25°
III	BI–C(OH)(H)–C(H)(OH)–CO CH_3	25°	VI	BI–(H)tetrahydrofuranone ring (O, =O)	100°
			VII	BI–C(H)(OH)–CH_2 CH CO_2H	237°

separated on keeping at the ambient temperature. The yield was 0.7 g (75 per cent). The colourless prisms obtained by recrystallization from isopropanol melted at 202—206° (Found: C 59.9, H 7.0, N 11.4. $C_{12}H_{16}N_2O$·HCl requires C 59.9, H 7.1, N 11.6). Thus the isolated product was the hydrochloride of the synthesized benzimidazole derivative. The free base was isolated as follows.

The hydrochloride (0.46 g) was dissolved in water (2 ml) and neutralized by adding ammonium hydroxide solution (2 M) dropwise. A white precipitate occurred which was collected by filtration and washed with water. The product was dissolved in hot methanol (2 ml). Colourless plates separated following the addition of water (0.5 ml). The yield was 0.35 g (89 per cent), m.p. 171—175°, $[\alpha]_D^{5} = -35.6°$ (C 0.50, hydrochloric acid 1 M) (Found: C 70.7, H 7.8, N 13.8. $C_{12}H_{16}N_2O$ requires C 70.6, H 7.9, N 13.7).

UV spectrum: methanol λ_{max} = 281, 274.5, 244 nm, log ϵ = 3.90, 3.90, 3.80

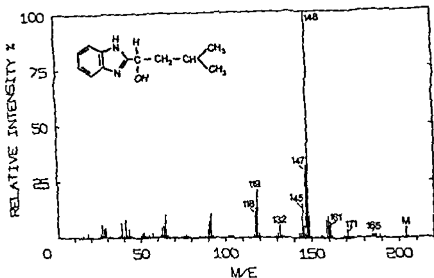


Fig 38 — Mass spectrum of (*S*) (—) 2 (1 hydroxy 3 methylbutyl)benzimidazole (Compound I)

categories were considered to be closely related structurally If this assumption is correct and if the ion pattern is indicative of this type of compound, it should be possible to use this observation to establish the position of the >CH O group in the alkyl chain of metabolite A

To ascertain whether this observation is also valid in cases of aliphatic substitution the mass spectra of a number of alkyl (2 benzimidazolyl)carbinols with various substituents in the alkyl chain were studied The compounds investigated

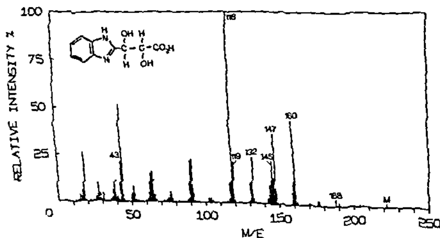


Fig 39 — Mass spectrum of (2*R* 3*S*) (+) 2 3 dihydroxy 3-(2 benzimidazolyl)propionic acid (Compound II)

(1S, 2S) (+)-2 (1 2 3 trihydroxypropyl)benzimidazole (IV)

Compound (III) (0 5 g) dissolved in absolute tetrahydrofuran (THF) (50 ml) was slowly added to an agitated suspension of lithium aluminium hydride (0 45 g) in absolute THF (30 ml) in a flask fitted with a condenser carrying a calcium chloride tube at the top The mixture was refluxed and agitated vigorously for 3 h Following the dropwise addition of hydrochloric acid (5 M 7 5 ml) the mixture was concentrated in a rotary evaporator at room temperature The residue was dissolved in water (50 ml) and treated with charcoal and celite The solut on was filtered and subsequently evaporated in a rotary evaporator at 40° The residue a thick mass was treated with sodium hydrogen carbonate (1 M) until no more precipitate was formed An equal volume of methanol was added and the slurry was heated almost to boiling A clarification of the solution was achieved by centrifugation yielding a clear solution and a sediment The latter was extracted with warm methanol and centrifuged The pooled methanol solutions were evap orated in a rotary evaporator and the residue was dissolved in water and treated with charcoal A crystalline precipitate consisting of colourless plates was separated on keeping at 0° during 10—12 h The plates were recovered by filtration and the yield was 0 2 g (50 per cent) The product melted at 189—190° after two recrystallizations from *n* butanol $[\alpha]_D^{25} = +49\ 3°$ (C 0 50 hydrochloric acid 1 M) (Found C 57 6 H 5 9 N 13 2 $C_{10}H_{12}N_2O_3$ requires C 57 7 H 5 8 N 13 5)

UV spectrum methanol λ_{max}=281 274 241 nm log ε=3 90 3 90 3 79

RESULTS AND DISCUSSION

The structural elucidation of metabolite A disclosed that in the course of the metabolism of FB a cleavage of the furan nucleus had taken place converting it to an open aliphatic chain The presence of a >CH O group was established by the NMR spectrum (δ=4 83 p 83) and by other means and it was considered necessary to demonstrate which carbon atom in the chain was carrying the hydroxyl group concerned

As can be seen from the mass spectrum of metabolite A (Fig 31) and as also mentioned briefly in Chapter 9 a number of ions were observed on fragmentation which might be able to contribute to the structural elucidation of the metabolite The ions concerned were *m/e* (148) 147 119 and 118 occurring also in the mass spectra of certain benzimidazole derivatives studied by Lawesson *et al* (1968) The compounds investigated by Lawesson and co workers included among others (2 benzimidazolyl)phenylcarbinol also known as (α hydroxybenzyl)benzimidazole (HBB) some derivatives of this compound with a substituted phenyl group and (2 benzimidazolyl)methylcarbinol The structures of these compounds all had in common a secondary hydroxyl-bearing carbon atom attached to the C 2 carbon of the benzimidazole moiety With regard to the s milarity between the mass spectra of metabolite A and the compounds studied by Lawesson *et al* the two

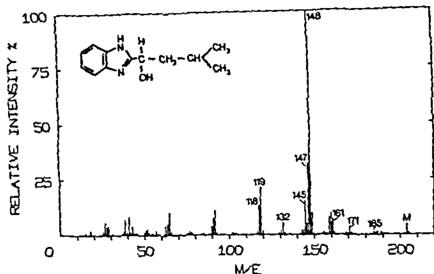


Fig 38 — Mass spectrum of (*S*) (—) 2 (1 hydroxy 3-methylbutyl)benzimidazole (Compound I)

categories were considered to be closely related structurally If this assumption is correct and if the ion pattern is indicative of this type of compound, it should be possible to use this observation to establish the position of the >CH O group in the alkyl chain of metabolite A

To ascertain whether this observation is also valid in cases of aliphatic substitution the mass spectra of a number of alkyl (2 benzimidazolyl)carbinols with various substituents in the alkyl chain were studied The compounds investigated

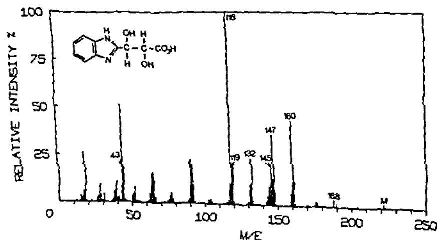


Fig 39 — Mass spectrum of (2*R*,3*S*) (+) 2 3 dihydroxy 3 (2 benzimidazolyl)propionic acid (Compound II)

(1S, 2S)-(+)-2-(1,2,3-trihydroxypropyl)benzimidazole (IV)

Compound (III) (0 5 g), dissolved in absolute tetrahydrofuran (THF) (50 ml), was slowly added to an agitated suspension of lithium aluminium hydride (0 45 g) in absolute THF (30 ml) in a flask fitted with a condenser carrying a calcium chloride tube at the top The mixture was refluxed and agitated vigorously for 3 h Following the dropwise addition of hydrochloric acid (5 M, 7 5 ml) the mixture was concentrated in a rotary evaporator at room temperature The residue was dissolved in water (50 ml) and treated with charcoal and celite The solution was filtered and subsequently evaporated in a rotary evaporator at 40° The residue, a thick mass, was treated with sodium hydrogen carbonate (1 M) until no more precipitate was formed An equal volume of methanol was added and the slurry was heated almost to boiling A clarification of the solution was achieved by centrifugation, yielding a clear solution and a sediment The latter was extracted with warm methanol and centrifuged The pooled methanol solutions were evaporated in a rotary evaporator, and the residue was dissolved in water and treated with charcoal A crystalline precipitate consisting of colourless plates was separated on keeping at 0° during 10—12 h The plates were recovered by filtration, and the yield was 0 2 g (50 per cent) The product melted at 189—190°, after two recrystallizations from *n* butanol $[\alpha]_D^{25} = +49\ 3°$ (C 0 50, hydrochloric acid 1 M) (Found C 57 6, H 5 9, N 13 2 $C_{10}H_{12}N_2O_3$ requires C 57 7, H 5 8, N 13 5)

UV spectrum methanol λ_{max}=281 274 244 nm log ε=3 90 3 90 3 79

RESULTS AND DISCUSSION

The structural elucidation of metabolite A disclosed that in the course of the metabolism of FB a cleavage of the furan nucleus had taken place converting it to an open aliphatic chain The presence of a >CH O group was established by the NMR spectrum (δ=4 83, p 83) and by other means and it was considered necessary to demonstrate which carbon atom in the chain was carrying the hydroxyl group concerned

As can be seen from the mass spectrum of metabolite A (Fig 34) and as also mentioned briefly in Chapter 9, a number of ions were observed on fragmentation, which might be able to contribute to the structural elucidation of the metabolite The ions concerned were *m/e* (148), 147 119, and 118, occurring also in the mass spectra of certain benzimidazole derivatives studied by Lawesson *et al* (1968) The compounds investigated by Lawesson and co-workers included among others (2 benzimidazolyl)phenylcarbinol also known as (α hydroxybenzyl)benzimidazole (HBB), some derivatives of this compound with a substituted phenyl group and (2 benzimidazolyl)methylcarbinol The structures of these compounds all had in common a secondary, hydroxyl bearing carbon atom attached to the C 2 carbon of the benzimidazole moiety With regard to the similarity between the mass spectra of metabolite A and the compounds studied by Lawesson *et al*, the two

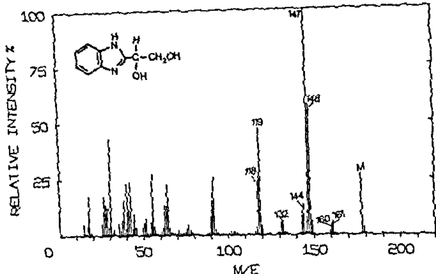


Fig 42 — Mass spectrum of (R) (—) 2 (1 2 dihydroxyethyl)benzimidazole (Compound V)

Ion *m/e* 147 (see (a) in Diagram 5, p 111) is formed by rupture of the 1,2 bond[1] (*cf* general formula in Table 17) The formation of this ion could be expected from what is known about the fragmentation of alkyl substituted aromatic compounds and secondary alcohols (Kraft & Spiteller 1968) and of diols (*cf*

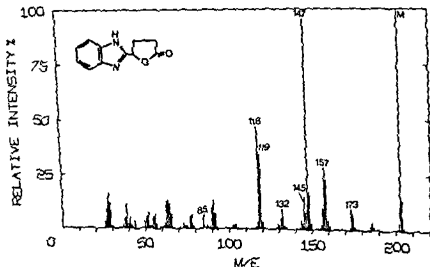


Fig 43 — Mass spectrum of (S) (+) 4-(2 benzimidazolyl) γ butyrolactone (Compound VI)

[1] An asterisk in the text or in Diagram 5 *is used* to indicate that a "metastable peak" has been observed for the fragmentation concerned

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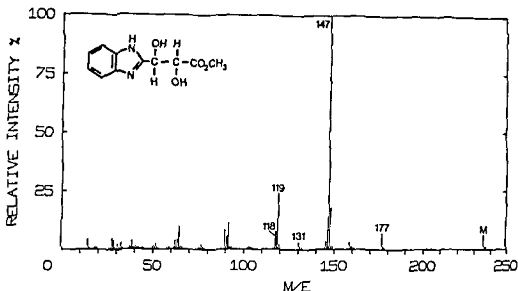


Fig 40 — *Mass spectrum of methyl (2R 3S) (+) 2 3 dihydroxy 3 (2 benzimidazolyl)pro*pionate (Compound III)

are shown in Table 17 In addition to these compounds compound VI was also examined Being a γ butyrolactone, it differed structurally from the others Figs 38—44 illustrate the mass spectra of the compounds investigated From these spectra it can be seen that the above mentioned feature, *i e*, the simultaneous occurrence of ions *m/e* 147, 119 and 118, is observed in all of these spectra In addition to these ions, the presence of the ion *m/e* 148 has been demonstrated in each case, and its intensity is high in some of the spectra

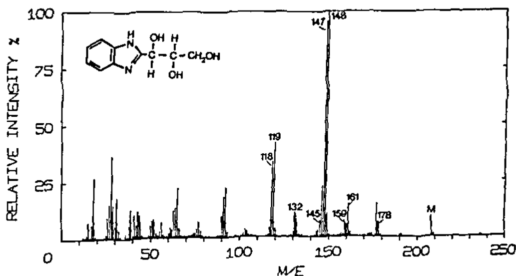


Fig 41 — Mass spectrum of (1S 2S) (+) 2 (1 2 3 trihydroxypropyl)benzimidazole (Compound IV)

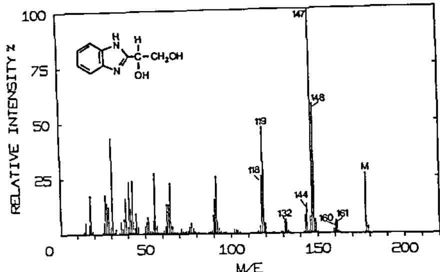


Fig 42 — Mass spectrum of (*R*) (—) 2 (1 2 dihydroxyethyl)benzimidazole (Compound V)

Ion *m/e* 147 (see (a) in Diagram 5, p 111) is formed by rupture of the 1,2 bond[19] (*cf* general formula in Table 17) The formation of this ion could be expected from what is known about the fragmentation of alkyl substituted aromatic compounds and secondary alcohols (Kraft & Spiteller 1968) and of diols (*cf*

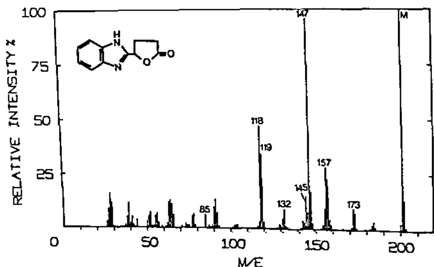


Fig 43 — Mass spectrum of (*S*) (+) 4 (2 benzimidazolyl) γ butyrolactone (Compound VI)

[19] An asterisk in the text or in Diagram 5 is used to indicate that a "metastable peak has been observed for the fragmentation concerned

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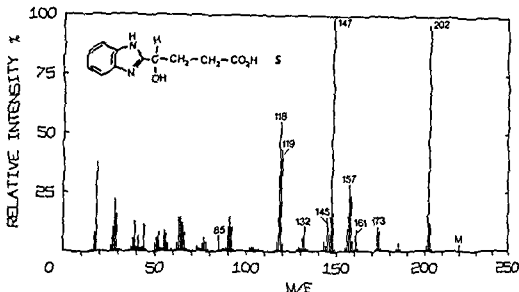


Fig 44 — Mass spectrum of synthetic (*S*) (—) 4 (2 benzimidazolyl) 4 hydroxybutyric acid (Compound VII)

Budzikiewicz *et al* 1967) The ion *m/e* 147 gives rise to the daughter ion *m/e* 119 (see (a) → (b) in Diagram 5) by loss of CO, as can be concluded from the metastable transition ($147^+ \rightarrow 119^+$) This cleavage was found in the spectra of all compounds investigated (*cf* Figs 38—44)

Ion *m/e* 148 (see (c) in Diagram 5) is formed by a McLafferty rearrangement, leading to cleavage of the 1,2 bond In cleavages of this type the hydrogen is transferred from an adjacent hydroxyl group (*e g* in compounds IV and V) or from an alkyl group (*e g*, *in compound I*) (*cf* Lawesson *et al* 1968, Nishiwaki 1968) to the aza nitrogen This ion in its turn gives rise to the ion *m/e* 147, as can be seen from the metastable transition ($148^+ \rightarrow 147^+$) Ion *m/e* 147 forms the daughter ion *m/e* 119 (*cf* (c) → (a) → (b) in Diagram 5)

Ion *m/e* 118, the benzimidazole radical ion (see (d) in Diagram 5), may have been formed directly from the molecular ion by rupture of the (C 2) 1 bond but other paths of fragmentation may also be followed

Lawesson *et al* (1968) assigned a protonated benzimidazole cation structure to the ion *m/e* 119 and in the case of ion *m/e* 147 it was stated that the composition corresponds to the appropriate protonated formyl benzimidazole cation

The composition of the ions discussed above has been confirmed by high resolution mass spectrometry (*cf* Table 18)

The fragmentation is somewhat different however in the case of compound VI (Fig 43) which is a 4 (2 benzimidazolyl) γ butyrolactone (BI lactone) The molecular peak is the base peak at *m/e* 202

Ion *m/e* 118, (d) in Diagram 5, arises from the molecular ion by rupture of the

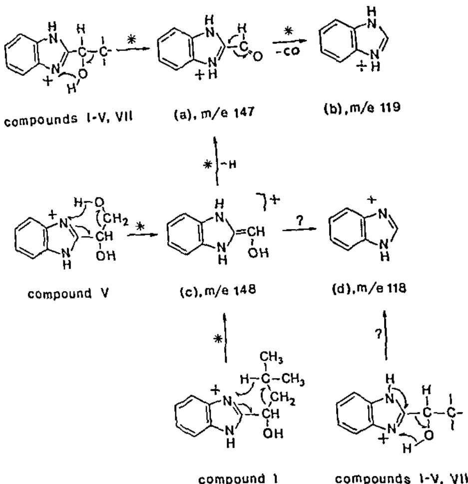


Diagram 5 — Characteristic patterns of cleavage and rearrangement of alkyl (2 benzimidazolyl)carbinols

(C 2) 1 bond with simultaneous hydrogen transfer from the lactone moiety to the benzimidazole moiety The lactone fragment formed by cleavage of the same bond but without hydrogen transfer gives rise to a peak of low intensity at *m/e* 85 Ion *m/e* 147 is formed according to the process ($202^+ \xrightarrow{*} 147^+$), which corresponds to loss of a C_3H_3O fragment from the lactone to give a protonated 2 benzimidazolyl CHO ion (*cf* Friedman & Long 1953 Mc Fadden *et al* 1965) The structure assigned to this ion and to (a) in Diagram 5, as observed in the spectra of compounds I—V (see above) has been confirmed by high resolution mass spectrometry and by the metastable transition ($147^+ \xrightarrow{*} 119^+$) resulting in the formation of the daughter ion (b) (Diagram 5)

Table 18 — Composition of some ions in the spectra of the compounds I II IV VI and VII

The plus sign indicates that only a singlet is observed under high resolution conditions and that it has a mass number consistent with the composition indicated

m/e	Composition	Compound				
		I	II	IV	VI	VII
85	$C_4H_5O_2$				+	+
118	$C_7H_6N_2$	+	+	+	+	+
119	$C_7H_7N_2$	+	+	+	+	+
147	$C_8H_7N_2O$	+	+	+	+	+
148	$C_8H_8N_2O$	+	+	+	+	+
161	$C_9H_9N_2O$					+
202	$C_{11}H_{10}N_2O_2$				+	+
204	$C_{12}H_{16}N_2O$	+				
208	$C_{10}H_{12}N_2O_3$			+		
220	$C_{11}H_{12}N_2O_3$					+
222	$C_{10}H_{10}N_2O_4$		+			

The molecular ion of compound VII (*i e*, metabolite A) (Figs 34 and 41) is *m/e* 220 Loss of water leads to formation of *m/e* 202 (=M-18) This ion, with the same composition as that of BI lactone (compound VI), probably arises from a thermal process, which explains why no metastable peak has been found for this reaction That the product formed is the BI lactone and that the fragmentation occurs in the main after the formation of the lactone, is substantiated by the fact that the mass spectra of compounds VI and VII (*cf* Figs 43 and 44) are almost identical and by the composition of the fragment ions (*cf* Table 18) Ion *m/e* 161 (=M-59) is formed by cleavage of the 2,3 bond This ion is characteristic of compound VII (metabolite A) and is missing in the spectrum of compound VI (BI lactone) (*cf* Figs 34, 44 and 43)

A detailed report of this study will be published elsewhere

SUMMARY

Mass spectra for six aliphatic-chain substituted alkyl (2 benzimidazolyl)carbinols are given Each spectrum exhibits a characteristic set of peaks at *m/e* 148, 147 119, and 118 The same ions have also been observed in the mass spectrum of 4 (2 benzimidazolyl)-γ butyrolactone (BI lactone)

A comparison of the mass spectra of the BI-lactone and one of the compounds investigated, metabolite A, has shown that the latter compound is converted, probably thermally, mainly into the BI lactone, which is then fragmented as stated above

The composition of the basic ions has been substantiated by exact mass measurements

General Summary

The use of 2-(2-furyl)benzimidazole (FB) as a seed disinfectant has been increasing considerably during recent years, with a concomitant broadening of the areas treated. This development necessitates the detection and determination of residual amounts of this fungicide in biological materials. The *aims of the present investigation* have been to develop an analytical method *for the detection and* determination of FB in biological materials of animal origin and to isolate and identify metabolites of quantitative importance in the urine, following administration of FB. It has also been the author's aim to elaborate a procedure for quantitative determination of metabolites present in urin and to compare the metabolite pattern in the urine of a number of mammal species.

An analytical method for the detection and determination of FB in biological material has been developed (Chapter 1), according to which the organ samples are extracted with a mixture of organic solvents. The purification of the FB-containing extract is effected by liquid-liquid partitioning between an organic solvent and water. FB is separated and isolated by means of TLC. The quantitative determination is then made by UV spectrophotometry of the TLC eluate. The yield of the method in analyzing blood plasma and liver samples was 75—90 per cent (at FB contents within the range 0.3—4.8 μg in each determination), and the coefficient of variation was ±6 per cent. The detection limit of FB in the TLC stage was 0.2—0.3 μg.

Analysis of organ samples from animals that had been given FB orally prove that the method works satisfactorily (Chapters 1 and 2).

The absorption of FB from the gastro-intestinal tract in the rat and dog is rapid (Chapter 2) and the biotransformation is accomplished so fast that at FB doses of 100 and 56 mg per kg body weight respectively the compound can no longer be detected in the plasma after 4—5 h, using the analytical method devised for this purpose. In the dog the plasma peak level was reached about 1.5—2 h following administration of FB as free base.

The rapid disappearance of FB from the blood plasma (dog, rat) and liver (rat) (Chapter 2) together with the observation that no FB is excreted in the urine unmetabolized illustrate the rapid metabolism of FB *in* the animal organism. Consequently analytical methods for detection of FB ingestion *in* animals cannot be based on procedures for determination of the original compound but on methods for evaluation of available FB metabolites. For this reason the present

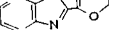
work has been primarily concerned with investigating the occurrence of FB metabolites in the urine

Urine samples from animals, following oral administration of FB, were divided into fractions by means of chromatography on a Sephadex column (Chapter 3) The presence of FB metabolites in the fractions was demonstrated utilizing the strong UV absorption and characteristic UV spectra associated with benzimidazoles A considerable number of metabolites have been detected by continuous recording of the UV absorption at 280 nm of the column eluates and by UV spectrophotometry of the collected fractions combined with TLC in various solvent systems The detection of the metabolites was facilitated by comparative investigations of the UV absorption properties of column eluates of urine samples from the experimental animals before and after treatment with FB The metabolites detected were designated by letters in the order of their elution in the column chromatography

Investigations of metabolite containing urine from the horse, goat, dog, rabbit, and rat (Chapter 3) demonstrated qualitative and quantitative variations in the metabolite excretion pattern Thus, the number of metabolites found varied from three in the rabbit to seven in the goat and rat The metabolites denoted A, B and E were found in the urine of each species studied in this investigation, in fact in considerable quantities in some of them They were consequently considered to be of greater analytical importance than the other metabolites

To be able to perform quantitative determinations of metabolites A, B, E (and Z) (Chapter 4) the compounds had to be prepared in pure form and characterized, including determinations of the molecular extinction coefficients of characteristic absorption bands in the UV range The excreted amount of each metabolite was calculated using the appropriate extinction coefficient following examination of each individual fraction by means of a recording UV spectrophotometer, in connection with the column chromatographic fractionation of the metabolite containing urine samples From these investigations it became obvious that metabolites A B, E (and Z) formed a considerable part of the metabolites observed These metabolites corresponded to 21—54 per cent of the FB dose given to the various species, as established by studying the excretion for 48 h (18 h in the horse), whereas the overall amount of the other metabolites was estimated to be only a few per cent It seems as though the metabolite pattern in the dog differs from that of the other species, the number of experimental animals does not however, allow any far reaching conclusions to be drawn In the dog, FB is excreted as metabolite A to a greater extent than in any of the other species investigated more than 90 per cent and 30—50 per cent, respectively, of the total amount metabolites excreted

The metabolites were separated and isolated from urine samples to obtain pure compounds for structural elucidation Metabolite Z was isolated from goat urine (Chapter 5) Horse urine was used for preparing concentrates of metabolites A, B and E, (Chapter 6) which were then employed in the isolation identification and characterization of the metabolites concerned



2 (2-furyl)benzimidazole (FB)

2 (2 furyl)-5(6) hydroxybenzimidazole (HOFB)

Metabolite Z

$C_6H_9O_6$ O

β-D-glucuronide of HOFB

Metabolite B

HO SO_2-O

sulphate ester of HOFB

Metabolite E

H_2C-CH_2

$-C$ CO

O OH

$\cdot CH_2$-CH_2-CO_2H

OH

(S)-(-)-4-(2-benzimidazolyl)-

-4-hydroxybutyric acid

Metabolite A

Diagram 6 — Metabolic pathways for 2 (2 furyl)benzimidazole in mammals (hypothetic intermediates are given in brackets)

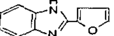
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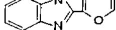
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O OH

H

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OH

(S)-(−)-4-(2-benzimidazolyl)--4-hydroxybutyric acid

Metabolite B

Metabolite E

Metabolite A

Diagram 6 — Metabolic pathways for 2 (2 furyl)benzimidazole in mammals (hypothetic intermediates are given in brackets)

In the biological degradation of FB according to the present investigation the enzymatic attack was directed either towards the benzene nucleus of the FB or towards its furan nucleus Diagram 6 illustrates the basic features of the metabolic pathways

The enzymatic hydroxylation of the benzene nucleus results in a phenol metab olite Z that was identified as 2 (2 furyl) 5(6) hydroxybenzimidazole (HOFB) (Chapter 5) This structure was also confirmed by synthesis

HOFB is mainly excreted as conjugates Metabolite B is the conjugate formed with glucuronic acid (Chapter 7) and metabolite E that with sulphuric acid (Chapter 8) The metabolites were identified by spectroscopy (UV IR NMR and mass spectroscopy) as well as by chemical and biochemical methods The structure of metabolite E was corroborated by a comparison of its spectroscopic and chemical properties with those of the synthetic sulphate ester of the phenol (HOFB)

Metabolite A was identified as (*S*) (—) 4 (2 benzimidazolyl) 4 hydroxybutyric acid on the basis of spectroscopic polarimetric and other data (Chapter 9) From this structure it may be concluded that the metabolite was formed by enzymatic attack on the furan nucleus of FB It is suggested that the attack is a hydroxy lation and a tentative pathway for the degradation including cleavage of the furan nucleus and formation of metabolite A is proposed

In order to determine the position of the alcoholic hydroxyl group in the aliphatic chain of metabolite A mass spectrometric studies of a series of 2 alkyl benzimidazole derivatives were carried out (Chapter 10) The compounds in vestigated were differently substituted in the alkyl chain but in each case the carbon in the alkyl chain attached to the C 2 carbon of benzimidazole was an alcoholic hydroxyl carrying carbon atom The mass spectra of all of these com pounds contained a characteristic set of peaks at *m/e* 148 147 119 and 118 and revealed the metastable transition ($147^+ \rightarrow 119^+$) As these characteristics were found in the mass spectrum of metabolite A as well the structural similarity be tween the metabolite and the compounds investigated was evident In this way the position of the hydroxyl bearing carbon was shown to be the C 4 carbon of the butyric acid

A synthesis based on the results of the structural investigation of metabolite A was carried out (Chapter 9) The product obtained was identical in every respect with metabolite A isolated from horse urine

The absolute configuration of metabolite A was established by investigations using circular dichroism (CD) measurements (Chapter 9) The compounds in vestigated included metabolite A the corresponding synthetic compound and benzimidazole derivatives of (*R*) (—) and (*S*) (+) lactic acid (*R*) (—) and (*S*) (+) mandelic acid and (*S*) (—) glyceraldehyde Since all of the reference compounds with *S*-configuration which were identical with respect to their absolute stereochemistry gave benzimidazole derivatives characterized by a negative CD maximum in the vicinity of 245 nm and since both metabolite A and the synthetic

compound showed such a negative CD maximum the *S*-configuration was allotted to both of them

Metabolite A has also been isolated from dog urine Complete identity between this metabolite and metabolite A isolated from horse urine has been established

As a result of the present investigation the detection and determination of FB can be accomplished in biological materials Since the main metabolites of FB in mammals have been isolated, and their structure and chemical properties elucidated, the ingestion of FB can be detected by means of its metabolites Furthermore, it may be claimed that these investigations offer an adequate basis for the development of a convenient method of analysis for the determination of FB metabolites in biological materials

The elucidation of the main metabolic pathways of FB in mammals shows in addition to hydroxylation of the benzene nucleus also the cleavage of the furan nucleus, probably following hydroxylation This is apparently the first time that this type of metabolism of a 2 substituted furan compound in mammals has been reported, and represents a contribution to increased understanding of the metabolism of furan compounds, the importance of which is emphasized by the direct or indirect exposure of human beings and animals to the wide variety of natural and synthetic products, drugs and pesticides containing the furan nucleus

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References

Andren, F Faltforsok rorande betning av vårvete 1965 och 1966 *Statens Växtskyddsanstalt, Medd* 1967, *13* 114, 485—493

Asmundson, C M, J Kumamoto & O E Smith The dry column chromatography of two isomeric abscisic acids *J Chromatog* 1969, *39*, 228—229

Austin, A T & J Howard The steric course of lactonisation following the deamination of glutamic acid, glutamine and γ aminovaleric acid *J Chem Soc* 1961, 3278—3284

Barker, S A, E J Bourne & D H Whiffen Use of infra red analysis in the determination of carbohydrate structure In, Methods of biochemical analysis Vol 3 Interscience New York, 1956, p 219

Beling C G Purification of urinary conjugated oestrogens by gel filtration *Nature* 1961, *192*, 326—327

Bellamy, L J The infra red spectra of complex molecules 2nd Ed, Methuen London 1958 p 174

Black, P J & M L Heffernan Proton magnetic resonance spectra of A_2B_2 systems *Australian J Chem* 1962, *15*, 862—864

Bloemendal, H Zone electrophoresis in blocks and columns Elsevier, Amsterdam London, New York, 1963, p 11

Borg, K Inverkan av betat utsade på viltfaunan Proc VIII Nord Veterinarmotet Helsingfors 1958 394—400

Borg, K, H Wanntorp, K Erne & E Hanko Alkyl mercury poisoning in the terrestrial Swedish wildlife *Viltrevy (Swedish Wildlife)* 1969, *6* 301—379

Bowker, D M & J E Casida Metabolism of the acaricide chemical, fenazaflor (5 6 dichloro 1 phenoxycarbonyl 2 trifluoromethylbenzimidazole), and related 2 trifluoromethylbenzimidazoles in certain mammals insects, and plants *J Agr Food Chem* 1969 *17* 956—966

Bowman, E R Studies on the excretion of 5 (3 pyridyl) tetrahydrofuranone 2 and its intermediary role in the metabolism of nicotine *Virginia J Sci* 1968 *19* 115—121

Brook, A J W & S Housley The interaction of phenols with Sephadex gels *J Chromatog* 1969, *41*, 200—204

Browning E Toxicity and metabolism of industrial solvents Elsevier Amsterdam, New York London, 1965

Budzikiewicz, H, C Djerassi and D H Williams Mass spectrometry of organic compounds Holden Day, San Francisco, 1967 pp 81 104 125

Burtis, C A, G Goldstein & C D Scott Fractionation of human urine by gel chromatography *Clin Chem* 1970, *16* 201—206

Cervinka, O, O Belovsky & P Rejmanova Asymmetrische reaktionen XXXIII Die absolute Konfiguration einiger Alkohole und Amine mit Pyridinring im Molekul *Z Chem* 1970 *10*, 69—70

Červinka, O & L Hub Asymmetric reactions XXVII Absolute configurations of , butyro

lactone γ carboxylic acid and γ valerolactone γ carboxylic acid *Collection Czech Chem Commun* 1968 33 2927—2932

Chilton W S & R C Krahn Rotatory dispersion of sugar heterocycles *J Am Chem Soc* 1967 89 4129—4132

Chilton W S & R C Krahn Rotatory dispersion of sugar heterocycles II Benzimidazoles and quinoxalines *J Am Chem Soc* 1968 90 1318—1323

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Crabbe P Optical rotatory dispersion and circular dichroism in organic chemistry Holden Day San Francisco London, Amsterdam 1965

Daly J W & D M Jerina Aerobic aromatic hydroxylation catalyzed by horseradish peroxidase Absence of NIH shift. *Biochim Biophys Acta* 1970 208 340—342

Daly J D Jerina & B Witkop Migration of deuterium during hydroxylation of aromatic substrates by liver microsomes I Influence of ring substituents *Arch Biochem Biophys* 1968 128 517—527

Determann, H Gelchromatographie Springer Verlag, Berlin Heidelberg, New York, 1967

Determann H & J Walter Source of aromatic affinity to Sephadex dextran gels *Nature* 1968 219 604—605

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Budzikiewicz H C Djerassi and D H Williams Mass spectrometry of organic c mpounds Holden Day San Francisco 1967 pp 81 104 125

Burtis C A G Goldstein & C D Scott Fractionation of human urine by gel chromato graphy *Clin Chem* 1970 *16* 201—206

Cervinka O O Belovsky & P Rejmanova Asymmetrische reaktionen XXXIII D e absolute Konfiguration einiger Alkohole und Amine mit Pyridinring im Molekul *Z Chem* 1970 *10* 69—70

Červinka O & L Hub Asymmetric reactions XXVII Absolute configurations of butyr

lactone γ carboxylic acid and γ valerolactone γ carboxylic acid *Collection Czech Chem Commun* 1968 33 2927—2932

Chilton, W S & R. C Krahn Rotatory dispersion of sugar heterocycles *J Am Chem Soc* 1967 89 4129—4132

Chilton, W S & R C Krahn Rotatory dispersion of sugar heterocycles II Benzimidazoles and quinoxalines *J Am Chem Soc* 1968 90 1318—1323

Conzelman G M Jr L E Flanders III K. Springer & D W Crout Beta glucuronidase sulfatase and phosphatase activities in the urine of dogs monkeys and mice *Lab Animal Care* 1970 20 192—197

Crabbe P Optical rotatory dispersion and circular dichroism in organic chemistry Holden Day San Francisco London, Amsterdam, 1965

Daly J W & D M Jerina Aerobic aromatic hydroxylation catalyzed by horseradish peroxidase Absence of NIH shift *Biochim Biophys Acta* 1970 208 340—342

Daly J D Jerina & B Witkop Migration of deuterium during hydroxylation of aromatic substrates by liver microsomes I Influence of ring substituents *Arch Biochem Biophys* 1968 128 517—527

Determann, H Gelchromatographie Springer Verlag, Berlin Heidelberg, New York, 1967

Determann, H & I Walter Source of aromatic affinity to Sephadex dextran gels *Nature* 1968 219 604—605

D mler R. J & K. P Link The characterization of lactic acid as the benzimidazole derivative *J Biol Chem* 1942 143 557—558

Dodgson K S Determ nation of inorganic sulphate in studies on the enzymic and non enzymic hydrolysis of carbohydrate and other sulphate esters *Biochem J* 1961 78 312—319

Elliott D C. In Data for biochemical research R. M C Dawson, D C Elliott, W H Elliott & K. M Jones (eds) Clarendon Press Oxford 1959 p 221

Fedoroňko M J Konigstein & K Linek Reaktion von o Phenylendamin mit Triosen *Collection Czech Chem Commun* 1968 33 2041—2048

Feigl F Spot tests Vol I Inorganic applications 4th Ed Elsevier Amsterdam, Houston, London New York, 1954 p 289

Folch, J M Lees & G H Sloane Stanley A simple method for the isolation and purification of total lipides from animal tissues *J Biol Chem* 1957 226 497—509

Frank, A Kemisk toxikologiska studier av ett kvicksilverfritt betningsmedel Proc X Nord Veterinarmotet Stockholm 1966 89—92

Friedman L & F A Long Mass spectra of six lactones *J Am Chem Soc* 1953 75 2832—2836

Frohberger P E & C Wiegand Seed disinfectant for fusarium. Ger 1 209 799 (Appl 14 May 1964 27 Jan 1966) Quoted in C.A 1966 64 14900 g, (Patent)

Gardner J A R K Brantley & H Sherman Isolation and identification of a metabolite of methyl 1 (butylcarbamoyl) 2 benzimidazolecarbamate in rat urine *J Agr Food Chem* 1968 16 1050—1052

Gelotte B Studies on gel filtration Sorption properties of the bed material Sephadex *J Chromatog* 1960 3 330—342

Gerarde H W Toxicology and biochemistry of aromatic hydrocarbons Elsevier Amsterdam New York, London 1960

Gibson D T G E Cardini F C. Maseles & R. E Kallio Incorporation of oxygen 18 into benzene by Pseudomonas putida. *Biochemistry* 1970 9 1631—1635

Gronowitz S G Sorlin B Gestblom & R. A Hoffman Proton magnetic resonance of furans Chemical shifts and substituent effects *Arkiv Kemi* 1963 19 483—497

Hearse D J A H Olavesen & G M Powell The preparation and characterization of a

series of ³⁵S labelled aryl sulphate esters for metabolic studies *Biochem Pharmacol* 1969, *18*, 173—180

Holtzman, J, J R Gilette & G W A Milne The metabolic products of naphthalene in mammalian systems *J Am Chem Soc* 1967, *89*, 6341—6344

Ito, R, H Otaka, T Nemoto & K Nagasawa Biochemical studies on heterocyclic nitrogen compounds I Metabolic products of benzimidazole *Nippon Univ J Med* 1961, *3* 141—150

Jerchel, D, H Fischer & M Kracht Zur Darstellung der Benzimidazole *Ann Chem* 1952, *575*, 162—173

Jerfy, A & A B Roy Sulphated derivatives of some hydroxypyridines *Australian J Chem* 1970, *23*, 847—852

Jerina, D M, J W Daly, B Witkop, P Zaltzman Nirenberg & S Udenfriend 1 2 naphthalene oxide as an intermediate in the microsomal hydroxylation of naphthalene *Biochemistry* 1969, *9*, 147—156

Jones, L H P, D D Leaver & A A Milne Toxic effects in sheep following the administration of 2 phenylbenzimidazole as an anthelmintic *Res Vet Sci* 1965, *6*, 316—323

Kadin, S B, H J Eggers & I Tamm Synthesis and virus inhibitory activity of D and L isomers of 2 (α hydroxybenzyl) benzimidazole *Nature* 1964, *201*, 639—640

Kapoor, M & E R Waygood Metabolism of benzimidazole in wheat I Formation of benzimidazole nucleotide *Can J Biochem* 1965, *43*, 153—164

Khym, J N & M Uziel Differences in the behavior of the purine and pyrimidine bases on dextran columns at different pH values *J Chromatog* 1970, *47*, 9—14

Kimmerle, G Farbenfabriken Bayer AG Unpublished results Quoted by Wegler 1970

Kitcher, J P & P W Trudgill Oxidation of furan 2 carboxylate to oxo glutarate by Pseudomonas putida F2 Studies of enzymology and electron transport *Biochem J* 1970 *118*, 55P—56P

Klyne, W & P M Scopes Optical rotatory dispersion and circular dichroism in structural and stereochemical studies *Farmaco* (Pavia), *Ed Sci* 1969, *24*, 760—784

Kolk, H & G Ljungberg The effect of disinfection on cereal seeds determined in laboratory experiments and emergence tests in soil *Statens Vaxtskyddsanstalt, Medd* 1967, *13* 113, 471—484 (in Swedish)

Kowalska, B Quantitative chromatography of aromatic amino acids on Sephadex G-10 *Acta Biochim Polon* 1969, *16* 141—150

Kraft, M & G Spiteller Über den stufenweisen Abbau sekundarer und tertiarer Alkohole im Massenspektrometer *Monatsh Chem* 1968 99 1839—1850

Lawesson, S O, G Schroll, J H Bowie & R G Cooks Electron impact studies XXII Mass spectra of substituted benzimidazoles *Tetrahedron* 1968 *24*, 1875—1888

Leandri, G, A Mangini, F Montanari & R Passerini Ricerche sugli eterociclici Spettri di assorbimento U V e proprietà cromoforiche — Nota I Imidazoli, benzimidazoli e fenil benzimidazoli *Gazz Chim Ital* 1955 *85* 769—839

Lemieux, R U & J C Martin Applications of empirical rules for optical rotation to problems of conformational analysis *Carbohyd Res* 1970 *13* 139—161

Loev, B & K M Snader 'Dry column chromatography A preparative chromatographic technique with resolvability of thin layer chromatography *Chem Ind* London 1965, 15—16

Loev, B & M Goodman Dry column chromatography A preparative chromatographic technique with the resolution of thin layer chromatography *Chem Ind* London, 1967 2026—2032

Mathieson, D W Interpretation of organic spectra Academic Press, London, New York, 1965, p 35

McFadden W H, E A Day & M J Diamond Correlations and anomalies in mass spectra [illegible]

Mc [illegible]

McManus, E C, F V Washko & D J Tocco Gastrointestinal [illegible] thiabendazole in ruminants *Am J Vet Res* 1966, *27*, 849—855

Merck & Co Substituted benzimidazoles Brit 966, 796 (19 Aug 1964), U.S (Appl 4 May 1960) Quoted in C.A, 1965, 62, 2780 a, (Patent)

Meth Cohn, O & M A Naqui A ready synthesis of dihydrobenzimidazoles *Chem Commun* 1967, 1157—1158

Moll, H Über die saure und alkalische Desaminierung der γ Aminovaleriansaure *Chimia* (Aarau) 1966, *20*, 426—428

Morgan, K J The infrared spectra of some simple benzimidazoles *J Chem Soc* 1961, 2343—2347

Nakanishi K Infrared absorption spectroscopy Holden Day, San Francisco, 1964

Neilson, D G ORD of α hydroxy amidines and their transition metal complexes In, R Bonnett & J G Davis (eds) Some newer physical methods in structural chemistry *Mass* spectrometry Optical rotatory dispersion and Circular dichroism. United Trade Press, London, 1967, p 190

Nir I Determination of glucuronic acid by naphthoresorcinol *Anal Biochem* 1964, 8, 20—23

Nishiwaki T Mass spectrometric studies on 2 alkylbenzimidazoles Some evidence for fragmentation via quinoxalinium ions *J Chem Soc (C)* 1968, 428—430

Nøddegaard E, T Hansen & A Nøhr Rasmussen *Afprøvning af plantebeskyttelsesmidler 1965—66 Tidsk Planteavl* 1968, *71*, 456—462

Pease, H L & J A Gardiner *Fluorometric and colorimetric procedures for determining* residues of benomyl *J Agr Food Chem* 1969, *17*, 267—270

Phillips, M A *The formation of 2 substituted benzimidazoles J Chem Soc* 1928, 2393—2399

Plieninger, H, G *Ege, R. Fischer & W Hoffmann* Synthese in 4 Stellung carbathoxy substituierter Cyclohexenone *Chem Ber* 1961 *94*, 2106—2124

Rabiger D J & M M Joullie The ionization constants, ultraviolet and infrared spectra of some substituted benzimidazoles *J Org Chem* 1964, *29*, 476—482

Raines, S & Cs A. Kovács The preparation and rearrangement of 2 allylbenzimidazole *J Heterocyclic Chem* 1967, 4, 305—308

Randinitis E J, M Barr, H C Wormser & J B Nagwekar Kinetics of urinary excretion of D (—) mandelic acid and its homologs I Mutual inhibitory effect of D (—) mandelic acid and its certain homologs on their renal tubular secretion in rats *J Pharm Sci* 1970, 59 806—812

Richtmyer N K & C S Hudson A benzimidazole rule for the determination of configuration of the aldonic acids and related compounds *J Am Chem Soc* 1942, *64*, 1612—1615

Robinson H J H C. Stoerk & O E Graessle Studies on the toxicologic and pharmacologic *properties of thiabendazole Toxicol Appl Pharmacol* 1965, *7*, 53—63

Rosenblum, C. D J Tocco & E E. Howe Complications in residue studies with radioisotop drugs In *Proc 2nd Ann* Oak Ridge *Radioisotope Conf*, Gatlinburg, Tenn, pp 58—62, April 19—22, 1964

Rosenfeld D A, J W Pratt, N K Richtmyer & C S Hudson New benzimidazoles from polyhydroxy acids *J Am Chem Soc* 1951, *73*, 5907—5908

series of ^{35}S labelled aryl sulphate esters for metabolic studies *Biochem Pharmacol* 1969, *18*, 173—180
Holtzman, J, J R Gilette & G W A Milne. The metabolic products of naphthalene in mammalian systems *J Am Chem Soc* 1967, *89*, 6341—6344
Ito, R, H Otaka, T Nemoto & K Nagasawa Biochemical studies on heterocyclic nitrogen compounds I Metabolic products of benzimidazole *Nippon Univ J Med* 1961, *3*, 141—150
Jerchel, D, H Fischer & M Kracht Zur Darstellung der Benzimidazole *Ann Chem* 1952, *575*, 162—173
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Jerina, D M, J W Daly, B Witkop, P Zaltzman Nirenberg & S Udenfriend 1 2-naphthalene oxide as an intermediate in the microsomal hydroxylation of naphthalene *Biochemistry* 1969, *9*, 147—156
Jones, L H P, D D Leaver & A A Milne Toxic effects in sheep following the administration of 2 phenylbenzimidazole as an anthelmintic *Res Vet Sci* 1965, *6*, 316—323
Kadin, S B, H J Eggers & I Tamm Synthesis and virus inhibitory activity of D and L-isomers of 2 (α hydroxybenzyl) benzimidazole *Nature* 1964, *201*, 639—640
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Khym, J N & M Uziel Differences in the behavior of the purine and pyrimidine bases on dextran columns at different pH values *J Chromatog* 1970, *47* 9—14
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Kolk, H & G Ljungberg The effect of disinfection on cereal seeds determined in laboratory experiments and emergence tests in soil *Statens Växtskyddsanstalt, Medd* 1967, *13* 113, 471—484 (in Swedish)
Kowalska, B Quantitative chromatography of aromatic amino acids on Sephadex G 10 *Acta Biochim Polon* 1969, *16* 141—150
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Lawesson, S O, G Schroll, J H Bowie & R G Cooks Electron impact studies XXII Mass spectra of substituted benzimidazoles *Tetrahedron* 1968 *24*, 1875—1888
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Lemieux, R U & J C Martin Applications of empirical rules for optical rotation to problems of conformational analysis *Carbohyd Res* 1970 *13* 139—161
Loev, B & K M Snader 'Dry column chromatography A preparative chromatographic technique with resolvability of thin layer chromatography *Chem Ind* London 1965 15—16
Loev, B & M Goodman Dry column chromatography A preparative chromatographic technique with the resolution of thin layer chromatography *Chem Ind* London 1967 2026—2032
Mathieson, D W Interpretation of organic spectra Academic Press London New York 1965, p 35

Schering AG Fungicidal carbamate Neth 6, 612, 877 (Appl 15 March 1967), Ger (Appl 14 Sept 1965) Quoted in C A, 1967, 67, 81496 z, (Patent)
Schumann, G Beitrag zur systemischen Wirkung fungizider Benzimidazolderivate als Getreidebeizmittel *Nachrbl Deut Pflanzenschutzdienst* (Berlin) 1968, *20*, 1—5
Sinha, S N & E R Gabrieli A simple method for simultaneous determination of benzoic and hippuric acids in biological fluids *Clin Chim Acta* 1967, *19*, 313—317
Stahl, E Dunnschicht Chromatographie Springer Verlag, Berlin, Gottingen, Heidelberg, 1962
Thierfelder, H & E Schempp Die spezifische Drehung des aktiven phenyl γ oxybuttersauren Natriums und die Reduktion der Benzoylpropionsaure im Korper *Physiol Chem* 1921, *114*, 94—100
Thierfelder, H & E Schempp Das Verhalten von Benzoylpropionsaure, Phenylathylalkohol und Phenoxylessigsaure im Korper des Menschen und Hundes *Arch Ges Physiol* 1917, *167*, 280—288
Tocco, D J, R P Buhs, H D Brown, A R Matzuk, H E Mertel, R E Harman & N R Trenner The metabolic fate of thiabendazole in sheep *J Med Chem* 1964, 7, 399—405
Tocco, D J, J R Egerton, W Bowers, V W Christensen & C Rosenblum Absorption metabolism and elimination of thiabendazole in farm animals and a method for its estimation in biological materials *J Pharmacol Exp Therap* 1965, *149*, 263—271
Tocco, D J, C Rosenblum, C M Martin & H J Robinson Absorption metabolism and excretion of thiabendazole in man and laboratory animals *Toxicol Appl Pharmacol* 1966, *9* 31—39
Trudgill, P W, The metabolism of 2 furoic acid by Pseudomonas F2 *Biochem J* 1969 *113*, 577—587
Vandenbelt J M & C M Shearer *Carygraph* 1962, *2*, 8 (Applied Physics Corp Monrovia Calif) Quoted by White 1964
Vogeler, K UV spektroskopische Bestimmung von Ruckstanden in Getreidekornern nach Andwendung von Voronit® *Pflanzenschutz Nachr Bayer* 1968 *21*, 326—330
Wegler, R (ed) Chemie der Pflanzenschutz und Schadlingsbekampfungsmittel Band 2 Fungizide, Herbizide, Naturliche Pflanzenwuchsstoffe Ruckstandsprobleme Springer Verlag Berlin, Heidelberg New York, 1970, p 125
Weidenhagen, R Eine neue Synthese von Benzimidazol Derivaten *Chem Ber* 1936 69 2263—2272
White, R G Irrelevant absorption in quantitative ultraviolet spectrometry In Progress in infrared spectroscopy, Vol II Plenum Press New York, 1964, 295—296
Williams, R T Detoxication Mechanism 2nd Ed Chapman & Hall London 1959 pp 191 *a*, 279 *b* 280 *c* 284 *d* 288 *e* 546 *f*
Wróbel, J T & K Gałuszko *Furan chemistry Part IV New observations in IR spectra of* monosubstituted furan derivatives *Roczniki Chem* 1969 *43* 769—778
Youden, W J Statistical methods for chemists John Wiley & Sons, Chapman & Hall London, 1951

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with special reference to the action of spasmolytic and analgesic drugs

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I Introduction

The sphincter at the junction of the common bile duct and duodenum, described by ODDI (*1887*), *regulates* the flow of bile into the intestine and is closed during fasting, thus allowing the bile to flow into the gallbladder (BERGH 1942) An unphysiological function of this sphincter may adversely affect the pressure conditions in the biliary tract and promote the development of pathological disorders there (IVY 1934)

Spastic *contraction of the* choledochoduodenal junction is seen in diseases of the biliary tract, and it may also be associated with pathological conditions in certain other organs (ARCHIBALD 1919, SNELL et al 1936, IVY & GOLDMAN 1939, DOUBILET & MULHOLLAND 1948) Drugs used for various indications may also produce changes in the tone *of the choledochoduodenal junction* Drugs *increasing* the tone of this region may raise the intrabiliary pressure and provoke biliary colics (BUTSCH et al 1936, CURRERI & GALE 1950)

The present study investigated the effects of different types of drugs on the biliary tract, especially on the choledochoduodenal junction *The study was made on the rabbit and its purpose was*

— to find out whether adrenergic blocking agents, viz phentolamine and propranolol, affect the choledochoduodenal junction,

— to compare the ability of papaverine on the one hand, and anticholinergic agents, such as atropine, N butyl hyoscine, tiemonium, oxyphencyclimine and tropenziline *on the other to relax the* experimentally induced neurogenic and myogenic spasm of the choledochoduodenal junction,

— to determine the changes in the tone of choledochoduodenal junction and common bile duct pressure produced by analgesics, *such as* morphine, pethidine, pentazocine, phenazocine and dextropropoxyphene, and by the antitussive dextromethorphan

Changes in blood pressure were also recorded in order to assess the tolerance and circulatory effects of drugs

II Review of the literature

ANATOMICAL AND PHYSIOLOGICAL CONSIDERATIONS OF THE BILIARY TRACT

Choledochoduodenal junction in the rabbit

The common bile duct of the rabbit enters the superior part of the duodenum, and the pancreatic duct joins the distal ascending limb of the duodenum, separately from the common bile duct (BENSLEY 1948, WALKER 1965) The sphincter of papillae, i e the circular closing muscle in the duodenal end of the common bile duct, and the duodenal portion of the common bile duct surrounded by both longitudinal and circular muscle fibres of duodenal origin can be distinguished at the rabbit's choledochoduodenal junction or sphincter of Oddi (WESTPHAL 1923) Fibres of both the parasympathetic vagus nerve and the sympathicus enter the biliary tract The vagus fibres and the fibres of the sympathetic nervous system join into a plexus which surrounds the biliary passages, the hepatic artery and the portal vein (WESTPHAL 1923, BENSLEY 1948)

Tone of choledochoduodenal junction and gallbladder

Both nervous and hormonal factors may affect the tone of the choledochoduodenal junction Stimulation of the vagus nerve has been found to increase, and that of the sympathetic fibres to decrease, the tone in the biliary tract (WESTPHAL 1923, JOHNSON 1941, PALLIN & SKOGLUND 1961) On the other hand, WESTPHAL (1923) found that during the stimulation of the sympathetic nerves of the rabbit the choledochoduodenal flow was reduced despite the dilatation of the duodenal portion of the common bile duct. He attributed this to a separate constriction of the sphincter of papillae due to sympathicus stimulation He also found that slight stimulation of the vagus nerve was accompanied by relaxation of the choledochoduodenal junction, whereas strong stimulation markedly increased its tone

The gallbladder and choledochoduodenal junction cooperate in that, when the gallbladder contracts, the choledochoduodenal junction is relaxed Earlier authors emphasized the role of the nervous system in the regulation of the tone of the choledochoduodenal junction (DOYON 1893, 1894, MELTZER 1917) However, its hormonal control is probably

II Review of the literature

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more important than the nervous control (BOYDEN 1938, MAGEE 1965) According to MAGEE (1965), the contraction of the gallbladder and relaxation of the choledochoduodenal junction can be explained on the basis of hormonal action The *role of extrinsic nerves is possibly only* to facilitate or depress the response of the target organs to the hormones

Cholecystokinin, from the mucosa of the small intestine, relaxes the choledochoduodenal junction (SANDBLOM et al 1935, CREMA et al 1962, MAGEE 1965). Gastrin from gastric antral mucosa and secretin from small-intestinal mucosa may also relax the junction in the same way as cholecystokinin, for all three substances have been assumed to act on the same receptors in different organs (GROSSMAN 1970)

The activity of the duodenal musculature may influence the tone of the choledochoduodenal junction A rise in the duodenal tone increases the resistance of the choledochoduodenal junction of the rat, guinea pig, rabbit and dog (BURGET 1925, LUETH 1931, RITTER 1956) According to LONG (1942) and KOZOLL & NECHELES (1942), the dog sphincter can act independently of the duodenal musculature In man it has been found that sphincter of Oddi can act independently of the duodenal musculature (REICH 1940, CAROLI et al 1960), and also that duodenal musculature can affect the sphincter function (McGOWAN et al 1938, CAROLI et al 1960)

The gallbladder has its own autonomous function, with spontaneous, rhythmical contractions Cholecystokinin has a gallbladder-contracting action Gastrin also contracts the gallbladder but it probably has no physiological significance in this respect (VAGNE & GROSSMAN 1968) Secretin augments the action of cholecystokinin on the gallbladder (STENING & GROSSMAN 1969) The vagus stimulation increases and that of sympathicus fibres decreases the tone of the gallbladder (BAINBRIDGE & DALE 1905-6, WESTPHAL 1923, PALLIN & SKOGLUND 1961) Severance *of the sympathetic* nerves leading to the biliary tract in the dog or rabbit accelerates the rate of emptying of the gallbladder (WESTPHAL 1923, JOHNSON 1941) The volume of human gallbladder increases after vagotomy (JOHNSON & BOYDEN 1952)

ACTION OF DRUGS ON THE INTESTINAL SMOOTH MUSCLE

The intestinal system has a complex innervation which regulates its automatism (KOSTERLITZ & LEES 1964) The effect of the so called neurotropic agents on the intestine is mediated by the nervous route, whereas the musculotropic agents affect *the smooth muscle directly* (BURNSTOCK & HOLMAN 1966) A drug may affect the intestine also

by releasing a *biologically active* substance in the system (MUSCHOLL 1964, HANSSON et al 1964, BURKS & LONG 1966)

When acetylcholine released from the nerve ending is combined with the receptor of the target organ, the permeability of the cell membrane undergoes changes Depolarization of cell membrane initiates propagating action potentials which, in muscle, cause contraction (BULBRING 1954, 1955, 1957) Hyperpolarization of the cell membrane, in its turn, *inhibits the production* of propagating action potentials and therefore depresses the activity (BOWMAN et al 1968) Acetylcholine may have a depolarizing effect on some cells and excite them while other cells may be hyperpolarized and inhibited (BOWMAN et al 1968)

The anticholinesterases prevent the destruction of acetylcholine by inhibiting cholinesterase Physostigmine and neostigmine have an anticholinesterase effect, but they also seem to release acetylcholine from *the parasympathetic nerve* ending (BEAVER & RIKER 1962, CARLYLE 1963, MATTILA & IDÄNPÄÄN HEIKKILA 1968)

Noradrenaline and adrenaline increase tissue activity by producing depolarization of the cell membrane, whereas their inhibitory effect is associated with hyperpolarization (BOWMAN et al 1968) The smooth musculature of the intestine has both alpha and beta receptors which, except for the sphincters, are subjected to a relaxing action of adrenergic agents (DOLLERY et al 1969) CREMA & BERTE (1963), on the basis of experiments with isolated choledochoduodenal junctions of the cat and the calf, assumed that there were, at the junction, alpha receptors which mediate contraction and beta receptors which mediate *relaxation The same finding was reported by* LIEDBERG & PERSSON (1970) from their study on cats

The adrenergic blocking agents inhibit the alpha and beta receptor effects of adrenergic drugs, and they also affect the smooth muscle directly (ÅBLAD et al 1967, DOLLERY et al 1969)

The action of analgesics on the smooth muscle varies from one species to another In man morphine increases the intestinal tone and inhibits peristalsis, whereas in the guinea pig and *rabbit only the* latter effect can be registered (SCHAUMANN 1954, DANIEL & BOGOCH 1958) Analgesics produce changes in the release of intestinal transmitter agents in addition to which they may act on the smooth muscle cell *directly (SCHAUMANN 1957,* PATON 1957, TRENDELENBURG 1957, CAIRNIE et al 1961, SZERB 1961, DANIEL 1964, COX & WEINSTOCK 1966)

The barium ion has a stimulating effect directly on the smooth muscle cell, and it also affects nervous structures *(AMBACHE 1949,* KUSCHINSKY & LULLMANN 1950, TOH 1951, EDLUND & LOHI 1952, KOSTERLITZ & ROBINSON 1958, TAKAGI & TAKAYANAKI 1962)

more important than the nervous control (BOYDEN 1938, MAGEE 1965) According to MAGEE (1965), the contraction of the gallbladder and relaxation of the choledochoduodenal junction can be explained on the basis of hormonal action The role of extrinsic nerves is possibly only to facilitate or depress the response of the target organs to the hormones

Cholecystokinin, from the mucosa of the small intestine, relaxes the choledochoduodenal junction (SANDBLOM et al 1935, CREMA et al 1962, MAGEE 1965) Gastrin from gastric antral mucosa and secretin from small intestinal mucosa may also relax the junction in the same way as cholecystokinin, for all three substances have been assumed to act on the same receptors in different organs (GROSSMAN 1970)

The activity of the duodenal musculature may influence the tone of the choledochoduodenal junction A rise in the duodenal tone increases the resistance of the choledochoduodenal junction of the rat, guinea pig, rabbit and dog (BURGET 1925, LUETH 1931, RITTER 1956) According to LONG (1942) and KOZOLL & NECHELES (1942), the dog sphincter can act independently of the duodenal musculature In man it has been found that sphincter of Oddi can act independently of the duodenal musculature (REICH 1940, CAROLI et al 1960), and also that duodenal musculature can affect the sphincter function (McGOWAN et al 1938, CAROLI et al 1960)

The gallbladder has its own autonomous function, with spontaneous, rhythmical contractions Cholecystokinin has a gallbladder-contracting action Gastrin also contracts the gallbladder but it probably has no physiological significance in this respect (VAGNE & GROSSMAN 1968) Secretin augments the action of cholecystokinin on the gallbladder (STENING & GROSSMAN 1969) The vagus stimulation increases and that of sympathicus fibres decreases the tone of the gallbladder (BAINBRIDGE & DALE 1905 6, WESTPHAL 1923, PALLIN & SKOGLUND 1961) Severance of the sympathetic nerves leading to the biliary tract in the dog or rabbit accelerates the rate of emptying of the gallbladder (WESTPHAL 1923, JOHNSON 1941) The volume of human gallbladder increases after vagotomy (JOHNSON & BOYDEN 1952)

ACTION OF DRUGS ON THE INTESTINAL SMOOTH MUSCLE

The intestinal system has a complex innervation which regulates its automatism (KOSTERLITZ & LEES 1964) The effect of the so called neuro tropic agents on the intestine is mediated by the nervous route, whereas the musculotropic agents affect the smooth muscle directly (BURNSTOCK & HOLMAN 1966) A drug may affe

by releasing a biologically active substance in the system (MUSCHOLL 1964, HANSSON et al 1964, BURKS & LONG 1966)

When acetylcholine released from the nerve ending is combined with the receptor of the target organ, the permeability of the cell membrane undergoes changes Depolarization of cell membrane initiates propagating action potentials which, in muscle, cause contraction (BULBRING 1954, 1955, 1957) Hyperpolarization of the cell membrane, in its turn, inhibits the production of propagating action potentials and therefore depresses the activity (BOWMAN et al 1968) Acetylcholine may have a depolarizing effect on some cells and excite them while other cells may be hyperpolarized and inhibited (BOWMAN et al 1968)

The anticholinesterases prevent the destruction of acetylcholine by inhibiting cholinesterase Physostigmine and neostigmine have an anticholinesterase effect, but they also seem to release acetylcholine from the parasympathetic nerve ending (BEAVER & RIKER 1962, CARLYLE 1963, MATTILA & IDÄNPÄÄN HEIKKILÄ 1968)

Noradrenaline and adrenaline increase tissue activity by producing depolarization of the cell membrane, whereas their inhibitory effect is associated with hyperpolarization (BOWMAN et al 1968) The smooth musculature of the intestine has both alpha and beta receptors which, except for the sphincters, are subjected to a relaxing action of adrenergic agents (DOLLERY et al 1969) CREMA & BERTE (1963), on the basis of experiments with isolated choledochoduodenal junctions of the cat and the calf, assumed that there were, at the junction, alpha receptors which mediate contraction and beta receptors which mediate relaxation The same finding was reported by LILDBERG & PERSSON (1970) from their study on cats

The adrenergic blocking agents inhibit the alpha and beta receptor effects of adrenergic drugs, and they also affect the smooth muscle directly (ÅBLAD et al 1967, DOLLERY et al 1969)

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RESPONSE OF THE CHOLEDOCHODUODENAL JUNCTION TO DRUGS

A normal functioning of the choledochoduodenal junction is important for the filling of the gallbladder and in the regulation of intra biliary pressure A disturbed nervous and hormonal control of the choledochoduodenal junction (WESTPHAL 1923, DOUBILET & MULHOLLAND 1948), and various drugs are among the factors which may produce changes in the tone of the junction Owing to dissimilarity of experimental conditions, the findings have not been parallel for all drugs

Adrenergic agents have been found to relax the choledochoduodenal junction (BURGET 1925, LUETH 1931, SHI 1933, BERGH 1942, STALPORT et al 1959, STILLE & HILFIKER 1962), but findings to the contrary have also been reported (REACH 1920, LUETH 1931, DOUBILET & COLP 1937, BERGH 1942, ERDMANN & HENNE 1953, MENGUY et al 1958) According to CREMA & BERTE (1963) adrenaline and noradrenaline contract the cat's isolated terminal bile duct whereas isoprenaline has a relaxing effect BENZI et al (1964), in their studies on dogs and cats, found that isoprenaline always relaxed the terminal bile duct in situ, whereas the action of adrenaline and noradrenaline was not distinctly parallel CREMA & BERTE (1963) reported that dichlorisoprenaline contracted the isolated terminal bile duct of cat and calf

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PRESSURE IN THE COMMON BILE DUCT AND THE EFFECT OF ANALGESICS

The peristaltic activity present in the common bile duct of both experimental animals and man may produce small rhythmic fluctuations in the common duct pressure (WESTPHAL 1923, DANIELS et al 1967) While bile accumulates in the common bile duct the gallbladder serves as a pressure regulator for the biliary passages (IVY 1934) Bile secretion is governed both by nervous and by humoural factors (BAYLISS & STARLING 1902, GUNTER et al 1950, FRITZ & BROOKS 1963, JORPES et al 1965, ZATERKA & GROSSMAN 1966, MORRIS et al 1967)

BUTSCH et al (1936) recorded intrabiliary pressures from 0 to 20 mmH-O in patients undergoing choledochotomy Depending on circum stances, the results of measurements have most frequently remained within the range of 0—120 mmH O (KIPP 1936, CURRERI & GALE 1950, JACOBSSON 1957) In patients with biliary dyskinesia DAHL IVERSEN et al (1958) recorded intrabiliary pressures of 80—180 mmH_2O

BUTSCH et al (1936) reported that a therapeutic dose of morphine raised the human intrabiliary pressure from 0—20 mmH_2O to 200—300 mmH O Pethidine raises the human intrabiliary pressure less than morphine but more than codeine (GAENSLER & McGOWAN 1950) Methadone has been found to raise the human intrabiliaty pressure less than morphine (GAENSLER & McGOWAN 1950) KJELLGREN (1960) reported an average rise of the human intrabiliary pressure of about 55 mmH O with morphine and about 35 mmH-O with pethidine

Reports on the level of intrabiliary pressure in dogs have varied in most cases within the range of 0—130 mmH-O (POTTER & MANN 1926, PARRY et al 1955, WILLIAMS 1967, HANSSON 1968) MENGUY et al (1958) recorded an intrabiliary pressure of 111 mmH-O in chole cystectomized dogs, against 71 mmH-O in normal experimental animals Morphine, pethidine, codeine and methadone produced a rise in the canine intrabiliary pressure (MENGUY et al 1958)

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— dextropropoxyphene hydrochloride (Orion Oy, Helsinki)
— dextromethorphan hydrobromide (Medipolar Oy, Oulu)
— heparin (Heparin Medica® inject, Medica Oy, Helsinki)

The doses quoted correspond to the amount of base unless otherwise specified Solutions were made by dissolving the relevant agents in distilled water

METHODS

Anaesthesia

The animals were anaesthetized by injecting single doses of 7 5 mg/kg pentobarbital sodium into the lateral, marginal ear vein until the corneal reflex disappeared A total of about 40 mg anaesthetic per kg body weight was required Ether was also used as the anaesthetic in flow studies with pentobarbital During the experiments the test animal's temperature was maintained at 37 0—38 5°C with the aid of a heatable operation table and an electric lamp

Preparation of animals

Constant ventilation was maintained by connecting the trachea into an automatic respirator (Beatmungspumpe nach Schuler, B Braun, Apparatebau) Blood pressure was recorded electromanometrically from the left carotid artery by »Physiograph Six» equipment (E & M Instrument Co, Inc, Houston, Texas) Coagulation of the blood was inhibited by injecting heparin solution into the arterial cannula A cannula connected to a buret was introduced into the left jugular vein to enable injection of drugs into the circulation Isotonic NaCl -mannitol solution was infused into the jugular vein at a rate of 6 ml/h to keep the venous cannula patent.

The abdomen was opened by an upper midline incision To measure the choledochoduodenal flow, a cannula was introduced into the bile duct, towards the duodenum The bile duct on the liver side of the cannula was drained to prevent distension While the flow was being studied the gastric pylorus was tied with thick thread to prevent gastric secretion from entering the intestine The common bile duct pressure was measured electromanometrically using T tube*) connected to the bile duct

Both perfusion and pressure recordings were carried out while the abdomen was open The open abdomen was covered with a compress treated with physiological saline

*) Portex Kehr's T tube, FG6, Portlands Plastics Ltd, Hythe, Kent

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A total of 195 male rabbits, body weight 1 9—2 7 kg, were used for the study All animals were obtained from the Mankkaa Research Laboratory of Orion, Medical Suppliers, Mankkaa, Finland Before the experiments the animals were kept for a few days in standardized conditions, with drinking water ab libitum Food was withheld for 18—20 hours before the experiment

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— pentobarbital sodium (Nembutal® inject, Abbott Labs, North Chicago)
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— barium chloride dihydrate (E Merck A G, Darmstadt)
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— l-adrenaline bitartrate (C H Boehringer & Sohn, Ingelheim am Rhein)
— dl isoprenaline sulphate (Ph Nordica)
— phentolamine methanesulphonate (Regitine® amp, Ciba A G, Basel)
— propranolol hydrochloride (ICI Pharmaceuticals, Macclesfield, Cheshire)
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blood stream after the spasmogenic agent An interval of 5 minutes between the injections was sufficient time for the choledochoduodenal flow to become stabilized

The common bile duct pressure was recorded electromanometrically by physiograph The sensitivity of the equipment was calibrated so as to permit pressure measurements up to 200 mmH_2O The Statham transducer of the electromanometer had been placed on a level with the T tube in the bile duct The drugs were injected into the circulation after the intrabiliary pressure had been stabilized, and pressures were recorded for a minimum of 30 minutes

EVALUATION OF RESULTS

The change produced by the drug studied in the choledochoduodenal flow was considered the criterion of its action on the tone of the choledochoduodenal junction

When drug action was studied in a choledochoduodenal junction that was not tonicized, the observations were compared with the initial value before drug administration The relaxing effect of spasmolytics was determined on the basis of the increase they produced in flow through the choledochoduodenal junction constricted with a spasmogenic agent The increase in flow after spasmolytic (flow/min after spasmolytic minus flow in the 5th minute after spasmogenic agent) was expressed as a percentage of the decrease in flow (flow/min before spasmogenic minus flow in the 5th minute after spasmogenic agent) produced by the spasmogenic agent and recorded before the administration of spasmolytic Significances were determined by comparing the change produced by the spasmolytic with that observed without spasmolytic (in the control series) at the respective time

Blood pressure changes were determined in terms of differences from the initial value In determining the significance of the blood pressure effects of spasmolytics, the control values used were the changes observed at respective times after administration of neostigmine or barium chloride alone

The effect on perfusion of the additional pentobarbital given during pentobarbital anaesthesia, of neostigmine and barium chloride was determined in 10 animals In the other cases the flow and common bile duct pressures were measured after each dose in 5—6 animals Each animal was subjected to an average of 3 tests

Mean values, and standard errors of the mean values, were calculated on the findings Student's t test was used to determine the statistical difference between two mean values

Flow and pressure measurements

The action of drugs on the tone of the choledochoduodenal junction was studied using the system illustrated by the diagram of Fig 1

Physiological saline was perfused by a tube from a Mariotte bottle into the bile duct After the animal had been prepared, the level of the Mariotte bottle was adjusted so as to make the volume of fluid perfused through the choledochoduodenal junction approximately 1 ml/min or approximately 15 drops/min The perfused fluid was collected through the cannula introduced into duodenum onto the mesh pan of a drop counter transducer and recorded by physiograph A branch of the tube from Mariotte's bottle was connected with the electro manometer of the physiograph The Statham transducer of the electro manometer was adjusted to the level of the choledochoduodenal junction It provided information on the pressure in the common bile duct

After the rate of perfusion had become stabilized, the drug to be studied was injected into the blood stream The drug action on the choledochoduodenal junction was manifested by a change in the rate of perfusion The volume of fluid perfused was reported per minute The mean value of the last 5 pre injection minutes was taken as the initial flow rate per minute After the injection of the drug the flow was recorded for a minimum of 30 minutes

A spasm of the choledochoduodenal junction was produced separately by neostigmine or barium chloride When their action on these experimental spasms was studied, the spasmolytics were injected into the

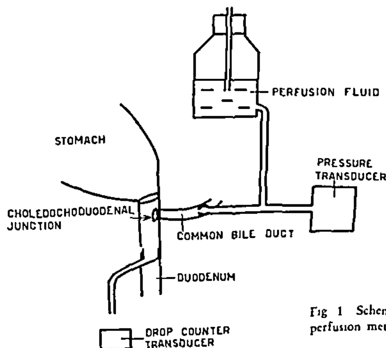


Fig 1 Schematic drawing of the perfusion method

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The effect on perfusion of the additional pentobarbital given during pentobarbital anaesthesia, of neostigmine and barium chloride was determined in 10 animals In the other cases the flow and common bile duct pressures were measured after each dose in 5—6 animals Each animal was subjected to an average of 3 tests

Mean values, and standard errors of the mean values, were calculated on the findings Student's t-test was used to determine the statistical difference between two mean values

Flow and pressure measurements

The action of drugs on the tone of the choledochoduodenal junction was studied using the system illustrated by the diagram of Fig 1

Physiological saline was perfused by a tube from a Mariotte bottle into the bile duct After the animal had been prepared, the level of the Mariotte bottle was adjusted so as to make the volume of fluid perfused through the choledochoduodenal junction approximately 1 ml/min or approximately 15 drops/min The perfused fluid was collected through the cannula introduced into duodenum onto the mesh pan of a drop counter transducer and recorded by physiograph A branch of the tube from Mariotte's bottle was connected with the electro manometer of the physiograph The Statham transducer of the electromanometer was adjusted to the level of the choledochoduodenal junction It provided information on the pressure in the common bile duct

After the rate of perfusion had become stabilized, the drug to be studied was injected into the blood stream The drug action on the choledochoduodenal junction was manifested by a change in the rate of perfusion The volume of fluid perfused was reported per minute The mean value of the last 5 pre-injection minutes was taken as the initial flow rate per minute After the injection of the drug the flow was recorded for a minimum of 30 minutes

A spasm of the choledochoduodenal junction was produced separately by neostigmine or barium chloride When their action on these experimental spasms was studied, the spasmolytics were injected into the

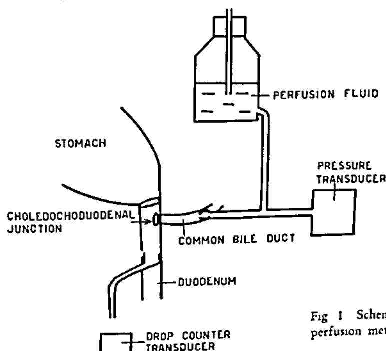


Fig 1 Schematic drawing of the perfusion method

blood stream after the spasmogenic agent. An interval of 5 minutes between the injections was sufficient time for the choledochoduodenal flow to become stabilized.

The common bile duct pressure was recorded electromanometrically by physiograph. The sensitivity of the equipment was calibrated so as to permit pressure measurements up to 200 mm H_2O. The Statham transducer of the electromanometer had been placed on a level with the T tube in the bile duct. The drugs were injected into the circulation after the intrabiliary pressure had been stabilized, and pressures were recorded for a minimum of 30 minutes.

EVALUATION OF RESULTS

The change produced by the drug studied in the choledochoduodenal flow was considered the criterion of its action on the tone of the choledochoduodenal junction.

When drug action was studied in a choledochoduodenal junction that was not tonicized, the observations were compared with the initial value before drug administration. The relaxing effect of spasmolytics was determined on the basis of the increase they produced in flow through the choledochoduodenal junction constricted with a spasmogenic agent. The increase in flow after spasmolytic (flow/min after spasmolytic minus flow in the 5th minute after spasmogenic agent) was expressed as a percentage of the decrease in flow (flow/min before spasmogenic minus flow in the 5th minute after spasmogenic agent) produced by the spasmogenic agent and recorded before the administration of spasmolytic. Significances were determined by comparing the change produced by the spasmolytic with that observed without spasmolytic (in the control series) at the respective time.

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The effect on perfusion of the additional pentobarbital given during pentobarbital anaesthesia of neostigmine and barium chloride was determined in 10 animals. In the other cases the flow and common bile duct pressures were measured after each dose in 5—6 animals. Each animal was subjected to an average of 3 tests.

Mean values and standard errors of the mean values, were calculated on the findings. Student's t test was used to determine the statistical difference between two mean values.

IV Effect of pentobarbital, neostigmine and barium chloride on the choledochoduodenal junction

For appraisal of the effect of pentobarbital, the anaesthetic used in the present work, on the choledochoduodenal junction, initial tests were made to determine the change it produced in the volume of fluid perfused through the choledochoduodenal junction This was done under both light ether and pentobarbital anaesthesia In the preliminary tests, the effect of neostigmine and barium chloride on the choledochoduodenal junction was also determined Changes in blood pressure were recorded at the same time

RESULTS

Changes in the choledochoduodenal flow

Pentobarbital sodium, 7 5 mg/kg, first produced in the ether-anaesthetized experimental animals a varying acceleration and retardation of about 15 per cent in the perfusion, but the flow stabilized itself in 10 minutes Also after the injection of additional pentobarbital during pentobarbital anaesthesia, similarly retarded and accelerated rates of perfusion were initially recorded In most cases the flow was slightly reduced but recovered its original rate in 10 minutes The changes produced by pentobarbital were not significant

Neostigmine (Fig 2), 0 2 μmol/kg, slowed down perfusion markedly in experimental animals under pentobarbital anaesthesia In the 2nd minute the flow was 85 and in the 5th minute 84 per cent smaller than its initial value ($p<0\ 001$) During the test the flow gradually increased, and in the 35th minute it was 22 per cent below its initial rate ($p<0\ 05$)

After injection of barium chloride (4 0 μmol/kg barium++) (Fig 2) the mean rate of perfusion remained significantly reduced ($p<0\ 001$) throughout the test In the 2nd minute it was 68 per cent ($p<0\ 001$) and in the 5th minute 61 per cent ($p<0\ 001$) lower than the initial rate The flow then increased and in the 35th minute it was, on an average 32 per cent ($p<0\ 001$) below the initial level

Changes in the arterial blood pressure

Pentobarbital sodium, dose 7 5 mg/kg, produced a distinct fall in blood pressure during both ether and pentobarbital anaesthesia In ether anaes-

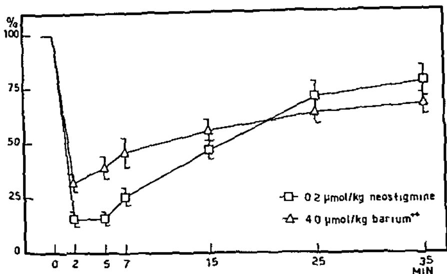


Fig 2 Choledochoduodenal flow in rabbits after the administration of neostigmine and barium (chloride) as a percentage of the initial flow (mean/minute in 5 pre injection minutes) The post injection flow during one minute was measured at given times Means ± S E M are given Ten animals in each group

thesia the blood pressure fell transiently by an average of 23 mmHg ($p<0\ 001$) and it was normal again in a few minutes In pentobarbital anaesthesia the blood pressure fell more heavily The average fall recorded at 30 seconds was 34 mmHg ($p<0\ 001$) After 10 minutes the blood pressure was lower than the initial level by an average of 9 mmHg ($p<0\ 01$)

Neostigmine (Table 1) produced an initial significant rise in blood pressure, by 21 mmHg In about 15 minutes the blood pressure had returned to the initial level

Barium chloride (Table 1) raised the blood pressure distinctly, by 23 mmHg After 15 minutes the rise was no longer significant

Table 1

Effect of *neostigmine and barium (chloride)* on rabbit blood pressure

Agent		Dose µmol/kg	Mean increase in blood pressure (mmHg±S E M)		
			1 min	5 min	15 min
Neostigmine	(10)	0 2	21±3 ***	7±4	1±4
Barium++	(10)	4 0	23±3 ***	16±3 ***	4±3

Number of animals in brackets. *** = $p<0\ 001$

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Changes in the arterial blood pressure

Pentobarbital sodium, dose 7 5 mg/kg, produced a distinct fall in blood pressure during both ether and pentobarbital anaesthesia In ether anaes-

V Effect of adrenergic and adrenergic blocking drugs on the choledochoduodenal junction

When the effect produced on the choledochoduodenal junction by drugs affecting the adrenergic system was studied, the agent used to block alpha receptors was phentolamine and that used to block beta receptors, propranolol Prior to this, the response of the choledochoduodenal junction to noradrenaline, adrenaline and isoprenaline, according to experimental conditions used, was determined

RESULTS

Changes in the choledochoduodenal flow

The effect of adrenergic agents on perfusion was studied using the doses of 2 0 and 8 0 μg/kg, and some additional experiments were made using doses of 0 5 μg/kg and 32 0 μg/kg

Noradrenaline tended to decrease the choledochoduodenal flow After the dose of 2 0 μg/kg, the flow rate per minute in two post-injection minutes was an average of 22 and 20 per cent below the initial level The changes, however, were not statistically significant

Adrenaline, in the doses used, had no distinct effect on perfusion taking place through the choledochoduodenal junction

Unlike the above two agents, isoprenaline increased perfusion in all experimental objects, the changes being significant during the first two minutes After the dose of 2 0 μg/kg (Fig 3) the flow in the lst and 2nd minutes was 26 per cent ($p<0\ 01$ and $p<0\ 05$) above the initial rate After the dose of 8 0 μg/kg the flow in lst and 2nd minutes showed a *significant* ($p<0\ 05$) *rate increase by 29 and 31* per cent

Phentolamine, 0 25 mg/kg, on the average tended to increase the rate of perfusion slightly During the 2nd minute perfusion was maximal and 28 per cent above the initial level After a dose of 1 0 mg/kg perfusion was found to slow down slightly During the 2nd minute it was, on the average, 27 per cent below the initial level However, the changes were not statistically significant

DISCUSSION

When LUETH (1931) studied the effect of various factors on the choledochoduodenal junction of the dog, he found that barbital was the most suitable anaesthetic He also found that a fall in blood pressure could be connected with an increase in the tone of the choledochoduodenal junction According to REYNELL & SPRAY (1957), anaesthesia induced with pentobarbital did not significantly affect the function of the rat's gastrointestinal tract A moderate to deep ether anaesthesia relaxes the smooth muscle of the human gastrointestinal tract (ORTH 1965) HICKEN (1951), however, failed to find any changes in tone produced by ether anaesthesia in the human sphincter of Oddi In the present study, when light ether anaesthesia was used, pentobarbital did not significantly affect the choledochoduodenal junction of the rabbit Nor was the additional dose of pentobarbital, given during pentobarbital anaesthesia, found to change the tone significantly In many experimental animals, however, a slight, transient retardation in perfusion was noted, presumably accompanying the heavy fall in blood pressure

Neostigmine, dose 0 2 μmol/kg, produced a strong spasm in the choledochoduodenal junction, as was reported also by KOZOLL & NECHELES (1942), and STALPORT et al (1959) This finding is probably associated with the neostigmine action transmitted by acetylcholine, which contracts the choledochoduodenal junction (CREMA & BENZI 1961) The dose of neostigmine used also raised the blood pressure This pressor effect of neostigmine is considered to arise from the stimulation of the sympathetic ganglia which e g agents like atropine have been found to inhibit (HILTON 1961, KHARKEVICH 1967)

The tone-increasing effect of barium is probably associated with its direct effect on the smooth muscle and nerve structures The contraction of intestinal smooth musculature produced by barium is probably also affected by acetylcholine, since barium has been found to release acetylcholine from the small intestine in vitro (AMBACHE 1946), while atropine also reduces the stimulating effect of barium (NECHELES et al 1953) Barium stimulates neurons at several sites, since e g hexamethonium, and atropine in the presence of hexamethonium, have been found to reduce the barium effect (EDLUND & LOHI 1952, FELDBERG 1951, KOSTERLITZ & ROBINSON 1958) In vasculature the barium ion produces vasoconstriction even when all nerves transmitting vasoconstriction are severed (NECHELES et al 1953)

V Effect of adrenergic and adrenergic blocking drugs on the choledochoduodenal junction

When the effect produced on the choledochoduodenal junction by drugs affecting the adrenergic system was studied, the agent used to block *alpha receptors* was phentolamine and that used to block beta receptors, propranolol Prior to this, the response of the choledochoduodenal junction to noradrenaline, adrenaline and isoprenaline, according to experimental conditions used, was determined

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The effect of adrenergic agents on perfusion was studied using the doses of 2 0 and 8 0 µg/kg, and some additional experiments were made *using doses of 0 5 µg/kg and 32 0 µg/kg*

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Adrenaline, in the doses used, had no distinct effect on perfusion taking place through the choledochoduodenal junction

Unlike the above two agents, isoprenaline increased *perfusion in all* experimental objects, the changes being significant during the first two minutes After the dose of 2 0 µg/kg (Fig 3) the flow in the 1st *and 2nd minutes was 26 per cent* ($p<0$ 01 *and* $p<0$ 05) above the initial *rate After* the dose of 8 0 µg/kg the flow in 1st and 2nd *minutes* showed a significant ($p<0$ 05) rate increase by 29 and 31 *per cent*

Phentolamine, 0 25 mg/kg, on the average *tended* to increase the rate of perfusion slightly During the 2nd minute *perfusion* was maximal and 28 per cent above the initial level After a dose of 1 0 *mg/kg* perfusion was found to slow down slightly During the 2nd *minute* it was, on the average, 27 per cent below the initial *level However*, the changes not statistically significant

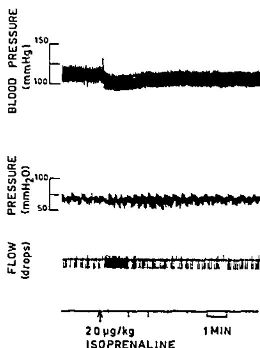


Fig 3

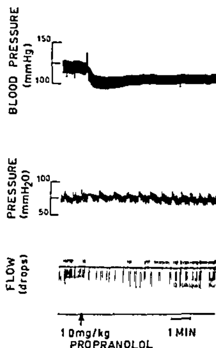


Fig 4

Effect of isoprenaline (Fig 3) and propranolol (Fig 4) on the choledochoduodenal flow common duct pressure and blood pressure in a rabbit

Propranolol retarded perfusion slightly and transiently After a dose of 0 25 mg/kg the flow in the 4th minute was 18 per cent below the initial level, and 1 0 mg/kg (Fig 4) produced a transient retardation in flow rate which in the 2nd minute was 24 per cent below the initial rate ($p < 0\ 05$)

Changes in the arterial pressure

Adrenergic agents, in doses of 2 0 and 8 0 µg/kg produced typical changes in blood pressure The rise produced by noradrenaline was 94—115 mmHg and that produced by adrenaline 49—91 mmHg The blood pressure fall caused by isoprenaline was 13—19 mmHg

Phentolamine (Table 2) produced a lasting fall in blood pressure, which after a dose of 1 0 mg/kg was maximally 38 mmHg

Propranolol (Table 2), dose 0 25 mg/kg, produced an acute transient rise in the blood pressure by 35 mmHg A fourfold dose produced a lasting fall in the blood pressure by a maximum of 20 mmHg

Table 2

Effect of phentolamine and propranolol on rabbit blood pressure

Drug		Dose mg/kg	Mean change in blood pressure (mmHg±S E M)			
			0 5 min	2 min	10 min	30 min
Phentolamine	(5)	0 25	—24±5 **	—28±5 ***	—25±5**	—24±6 **
"	(5)	1 0	—33±6 **	—35±6 ***	—37±6 ***	—38±6 ***
Propranolol	(6)	0 25	+35±5 ***	+ 1±3	— 8±4	— 5±3
"	(5)	1 0	—16±4 *	—18±4 **	—20±4 **	—19±4 **

Number of animals in brackets. * = p<0 05 ** = p<0 01, *** = p<0 001

DISCUSSION

Adrenaline, in the doses used, was not found to have any distinct effect on the choledochoduodenal junction of the rabbit Noradrenaline, on the average, reduced the choledochoduodenal flow but the change was not statistically significant It is possible that noradrenaline constricts the sphincter of papillae but this action is weakened by its relaxing effect on the duodenum CREMA & BERTE (1963) found that adrenaline and noradrenaline contracted the cat's isolated terminal bile duct, while LIEDBERG & PERSSON (1970) discovered the same response in the cat's choledochoduodenal junction in situ BENZI et al (1964), in their turn, reported that adrenaline and noradrenaline generally relaxed canine and feline terminal bile duct Isoprenaline, in the rabbit, relaxed the choledochoduodenal junction, and the same has been reported for the dog and cat from in situ studies (BENZI et al 1964, LIEDBERG & PERSSON 1970)

Phentolamine did not significantly affect the choledochoduodenal junction, although 0 25 mg/kg, on the average, slightly increased perfusion, a finding possibly associated with reduction in the alpha-adrenergic tone A dose of 1 0 μg/kg produced a short term retardation in perfusion Besides producing an alpha-blockade the imidazoline derivatives, such as phentolamine and tolazoline, directly stimulate the intestinal smooth musculature (AHLQUIST et al 1947, GOWDEY 1948, MAXWELL 1965)

Propranolol, on the other hand, proved to have a short-term retarding effect on perfusion, which may perhaps be associated with the blockade of beta adrenergic receptors, in other words, the situation would be analogous to that produced by propranolol in the bronchial smooth musculature (FITZGERALD 1969)

The haemodynamic effects of beta-blockade depend, among other

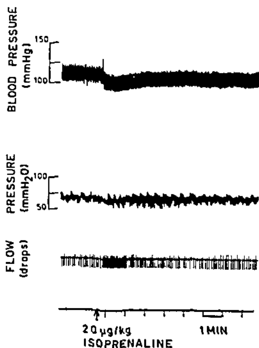


Fig 3

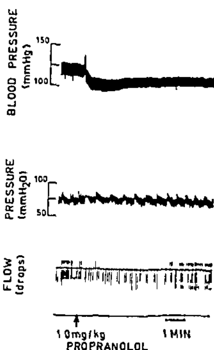


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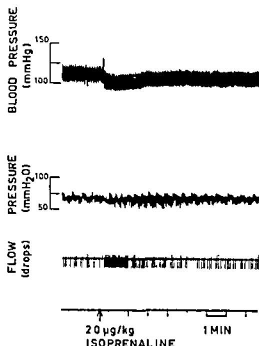
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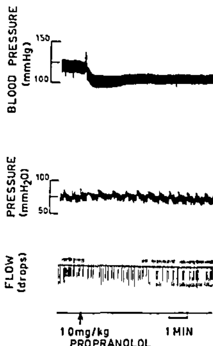


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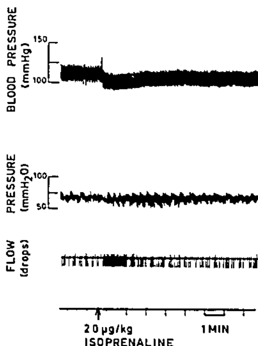
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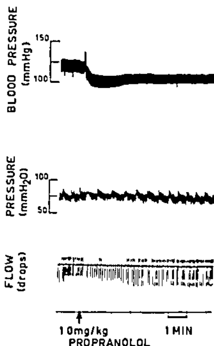


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»	(5)	1 0	−16±4 *	−18±4 **	−20±4 **	−19±4 **

Number of animals in brackets   * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

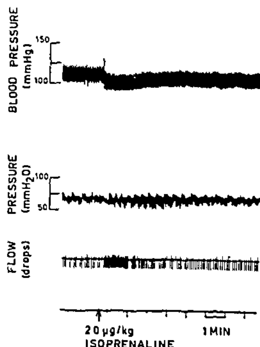
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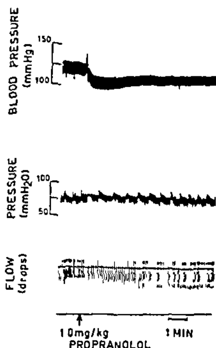


Fig 3 Fig 4

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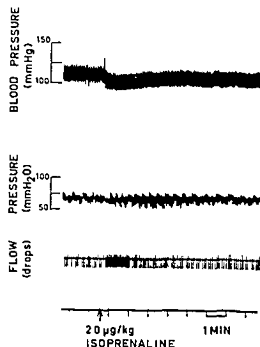


Fig 3

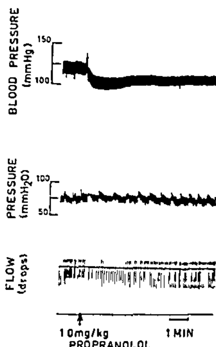


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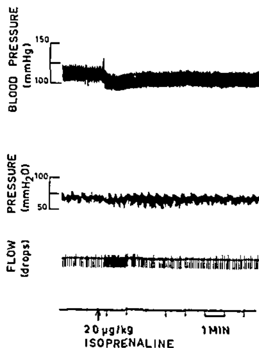


Fig 3

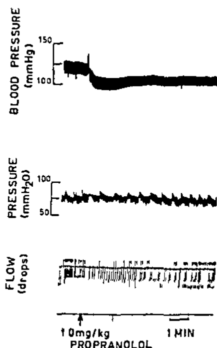


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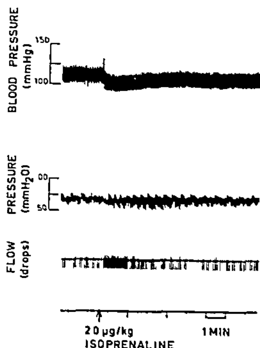


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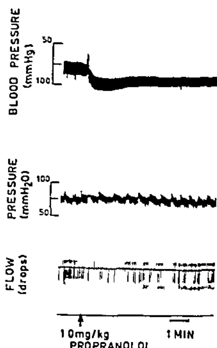


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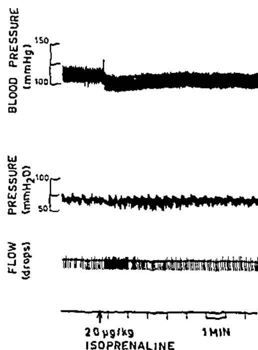
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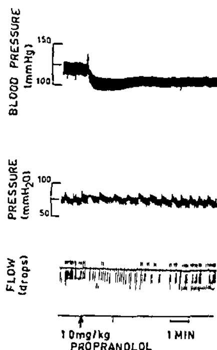


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things, on the prevailing sympathetic tone and on the potency and dose of the drug used (FITZGERALD 1969) Usually the agents producing beta blockade, such as propranolol, with no intrinsic sympathomimetic activity, cause the blood pressure to fall (DOLLERY et al 1969) This finding was also made with the present experimental arrangements when a dose of 1 0 mg/kg was used The blood pressure rise of short duration recorded after a propranolol dose of 0 25 mg/kg may have been due to a blockade of the beta-receptors mediating peripheral vasodilatation and/or reflex vasoconstriction in association with cardiac depression

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The present findings concerning adrenergic and adrenergic blocking agents seemed to suggest that there were, in the choledochoduodenal junction of the rabbit, beta-receptors mediating relaxation and possibly also alpha-receptors mediating contraction

VI Effect of spasmolytics on the neurogenic spasm of the choledochoduodenal junction

This section reports on studies of the ability of spasmolytics to relax a neurogenic spasm, induced by neostigmine, of the choledochoduodenal junction Before the administration of spasmolytics, given 5 minutes after 0 2 µmol/kg neostigmine, an average of 80—89 per cent of the choledochoduodenal flow was inhibited At the same time the action of spasmolytics on blood pressure was also determined

The relaxing effect of spasmolytics was determined on the basis of the increase they produced in the choledochoduodenal flow

RESULTS

Spasmolytic induced increase in the choledochoduodenal flow inhibited by neostigmine

The results (increase in flow) are given as a percentage of the decrease in flow produced by neostigmine and recorded before the administration of spasmolytic

Atropine (Fig 5), in an intravenous dose of only 0 0625 µmol/kg, proved to have a relaxing effect on the neostigmine induced spasm of the anaesthetized animals' choledochoduodenal junction The flow increase in two minutes recorded after this dose was 54 per cent ($p<0\ 01$) The effect of this dose was no longer significant later in the experiment A fourfold dose of atropine at first increased the perfusion markedly The flow change in two minutes was on an average 143 per cent ($p<0\ 001$) and after 10 minutes 76 per cent ($p<0\ 05$) but subsequently the observations did not differ significantly from the control values

N butyl hyoscine (Fig 6), dose 0 25 µmol/kg, produced a transient increase in the choledochoduodenal flow The flow change recorded in two minutes was 96 per cent ($p<0\ 001$) After a dose of 1 0 µmol/kg the flow remained at a significantly increased level throughout the experiment Flow increase in two minutes was 135 per cent ($p<0\ 001$) and after 30 minutes it was still 109 per cent ($p<0\ 05$)

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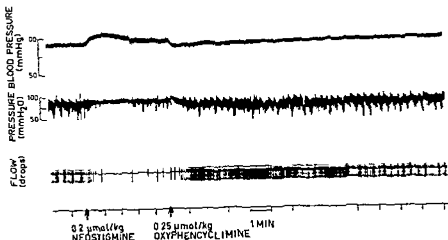


Fig 9 Effect of oxyphencyclimine given 5 min after neostigmine on the choledochoduodenal flow common duct pressure and blood pressure in a rabbit

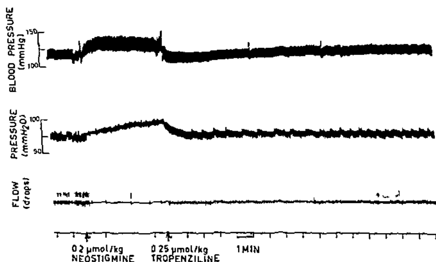


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dochoduodenal flow The dose of 16 0 µmol/kg produced a transient, significant increase in the choledochoduodenal flow The flow change after two minutes was 91 per cent ($p < 0.001$)

Oxyphencyclimine in a dose of 0 0625 µmol/kg was found to relax the spastic choledochoduodenal junction The flow changes recorded after

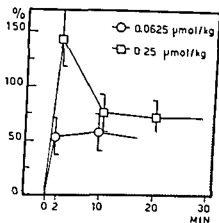


Fig 5

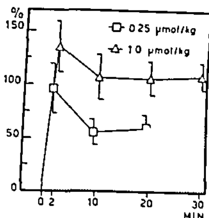


Fig 6

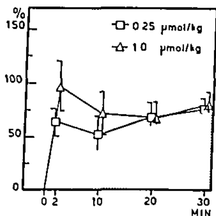


Fig 7

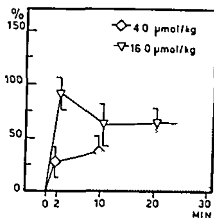


Fig 8

Increase of the choledochoduodenal flow in rabbits after the administration of atropine (Fig 5), N butyl hyoscine (Fig 6), tiemonium (Fig 7) and papaverine (Fig 8) given 5 min after neostigmine 0 2 µmol/kg The flow during one minute was measured at given times and the increase in flow was calculated as a percentage of the decrease in flow due to neostigmine, recorded before the administration of the spasmolytic Shaded area respective increase in flow without spasmolytic Means ± S E M are given 5—6 animals in each group

Tiemonium (Fig 7), dose 0 25 µmol/kg, produced a transient increase in the choledochoduodenal flow The change in two minutes after this dose was 64 per cent ($p<0\ 001$) Flow increase recorded two minutes after a tiemonium dose of 1 0 µmol/kg was 97 per cent ($p<0\ 001$) and 10 minutes after the dose 72 per cent ($p<0\ 05$) but later the recordings did not differ significantly from the control values

Studies of the relaxing effect of papaverine (Fig 8) on a neurogenic spasm of the choledochoduodenal junction required considerably larger doses than those listed above to increase the choledochoduodenal flow A papaverine dose of 4 0 µmol/kg had no significant effect on the chole-

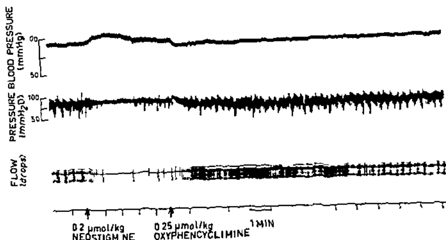


Fig 9 Effect of oxyphencyclimine given 5 min after neostigmine on the choledocho duodenal flow common duct pressure and blood pressure in a rabbit

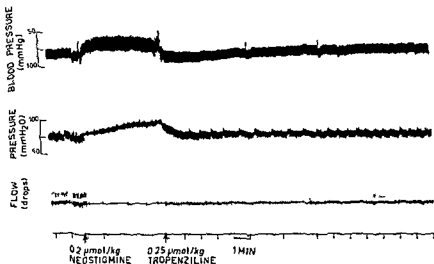


Fig 10 Effect of tropenziline given 5 min after neostigmine on the choledochoduodenal flow common duct pressure and blood pressure in a rabbit

dochoduodenal flow The dose of 16.0 µmol/kg produced a transient, significant increase in the choledochoduodenal flow The flow change after two minutes was 91 per cent ($p<0.001$)

Oxyphencyclimine in a dose of 0.0625 µmol/kg was found to relax the spastic choledochoduodenal junction The flow changes recorded after

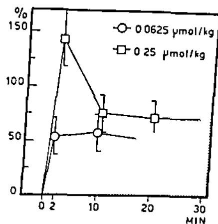


Fig 5

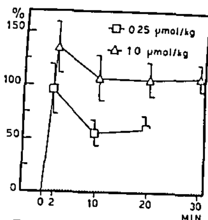


Fig 6

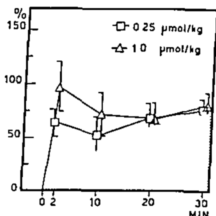


Fig 7

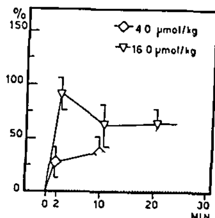


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Changes in the arterial blood pressure

Neostigmine, dose 0 2 μmol/kg, raised the blood pressure by 21 mmHg In about 15 minutes the blood pressure returned to its initial level

Certain anticholinergic agents given after neostigmine reduced the blood pressure The blood pressure changes produced by spasmolytics are shown in Table 3

Atropine doses of 0 0625 and 0 25 μmol/kg produced no significant changes in blood pressure

The maximum blood pressure fall recorded after a N butyl hyoscine dose of 0 25 μmol/kg averaged 10 mmHg and after a four times larger dose 18 mmHg

The maximum blood pressure fall recorded after a N butyl hyoscine dose of 0 25 and 1 0 μmol/kg were 10 and 19 mmHg respectively

Oxyphencyclimine and papaverine in the doses used had no significant act on on the blood pressure

Tropenziline, dose 0 25 μmol/kg, produced a fall of 10 mmHg in blood pressure which with a four times larger dose fell by an average of 16 mmHg

Table 3

Changes in rabbit blood pressure after administration of various spasmolytics Spasmolytics were given 5 min after neostigmine 0.2 μmol/kg

Drug		Dose μmol/kg	Mean change in blood pressure (mmHg±S E M)		
			0 5 min	2 min	10 min
Atropine	(5)	0 0625	—1±1	— 5±1	—3±6
»	(5)	0 25	—2±2	— 5±2	—8±3
N butyl hyoscine	(5)	0 25	—1±1	—10±3	—6±4
»	(5)	1 0	—8±2 **	—18±6 **	—7±8
Tiemonium	(6)	0 25	—2±1	—10±3	—5±2
»	(5)	1 0	—7±2 **	—19±4 **	—9±3
Oxyphencyclimine	(5)	0 0625	±0±1	— 8±3	—1±7
»	(5)	0 25	—2±1	— 8±2	—4±2
Tropenziline	(5)	0 25	—4±1 **	—10±1 **	±0±2
»	(6)	1 0	—7±2 **	—16±2 ***	—1±6
Papaverine	(5)	4 0	+3±2	+ 2±3	—3±4
»	(5)	16 0	—3±2	— 4±3	±0±3

Number of animals in brackets Statistical significances were calculated by comparing the changes in blood pressure observed after spasmolytics with the respective changes after neostigmine alone
** =p<0 01, *** = p<0 001

two and 10 minutes were 67 ($p<0.001$) and 83 per cent ($p<0.05$) respectively. A dose of 0.25 µmol/kg (Fig. 9) of oxyphencyclimine changed the perfusion markedly. The flow increase recorded after two minutes was 121 per cent ($p<0.001$) and after 30 minutes it was even 127 per cent ($p<0.05$).

The flow increases recorded after a tropenziline dose of 0.25 µmol/kg (Fig. 10) were 74 (<0.001) and 72 per cent ($p<0.05$) after two and 10 minutes respectively. A four times larger dose of tropenziline increased perfusion markedly: the change in flow recorded after two minutes was 131 per cent ($p<0.001$) and after 30 minutes it was still 120 per cent ($p<0.05$).

The action of most spasmolytic doses reached its maximum in the 2nd minute after their administration. For comparing the potencies of spasmolytics at that time, the log dose/response lines were drawn on the basis of two values for each spasmolytic (Fig. 11). These lines were used to determine which doses almost completely restored the choledochoduodenal flow previously reduced by neostigmine, i.e. 90 per cent of the flow rate (ED_{90}). These ED_{90} doses were: atropine 0.11, N-butyl hyoscine 0.20, tiemonium 0.75, oxyphencyclimine 0.12, tropenziline 0.44 and papaverine 15.60 µmol/kg.

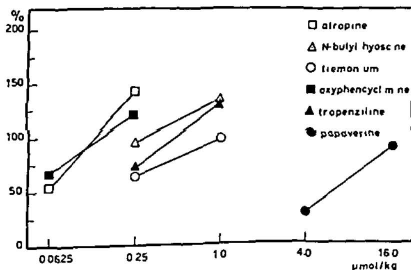


Fig. 11. Effect of spasmolytics on the choledochoduodenal flow inhibited by neostigmine. Log dose/response lines. The flow was measured during the 2nd minute after the administration of each spasmolytic. Increase in flow recorded after each spasmolytic was compared with the decrease in flow due to neostigmine observed before the administration of the spasmolytic.

Changes in the arterial blood pressure

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DISCUSSION

The effects of various spasmolytics on the choledochoduodenal junction treated with neostigmine were compared It was found that atropine and oxyphencyclimine were the most effective antispasmodics FINKELSTEIN et al (1959) reported that atropine and oxyphencyclimine had an approximately equal action on the acetylcholine-induced spasm of rabbit ileum The spasmolytic action of atropine proved to be of shorter duration than that of oxyphencyclimine This may partly also be due to the experimental animal's ability to inactivate atropine at a higher rate with the enzyme atropine-esterase (KRANTZ & CARR 1961)

In the present experiments neostigmine raised the arterial blood pressure, apparently because it stimulated sympathetic ganglia (KHARKEVICH 1967) With the doses used, neither atropine nor oxyphencyclimine affected the blood pressure significantly Hence their effect on the sympathetic ganglia would seem to be small in doses which already reveal a distinct spasmolytic action HILTON (1961) found that an atropine dose of 1 mg/kg in dogs affected significantly the pressor effect of neostigmine FINKELSTEIN et al (1959), who examined anaesthetized cats, reported that oxyphencyclimine did not block the sympathetic ganglia until the doses had been increased to 3—10 mg/kg intravenously

LECCHINI et al (1969) compared the actions of atropine, tropenziline and N-butyl hyoscine on isolated guinea pig colon They found that atropine had the highest and N butyl hyoscine the lowest anticholinergic activity WICK (1951) found that N butyl hyoscine had a good inhibitory effect on the nicotinic actions of acetylholine In the present study the relaxing effect of N-butyl hyoscine on the neostigmine induced spasm of rabbit's choledochoduodenal junction was also weaker than that of atropine, but better than that of tropenziline In the rabbit, the inhibitory effects on the sympathetic ganglia of N butyl hyoscine and tropenziline were of the same magnitude appraised on the basis of the fall they produced in blood pressure

The effect of atropine (ED_{90}) on the choledochoduodenal flow inhibited by neostigmine was about seven times stronger than that of tiemonium The action of atropine on the acetylcholine induced spasm of isolated guinea pig ileum has been reported to be about 10 times stronger than that of tiemonium (DUCHENE-MARULLAZ et al 1963) The antispasmodic effect of tiemonium was found by DUCHENE MARULLAZ et al (1963) to exceed that of papaverine, while tiemonium action on the intestinal motility exceeded that of atropine A tiemonium dose of 1 0 μmol/kg produced a relatively heavy fall in blood pressure, apparently indicating that this dose sufficed to inhibit cholinergic transmission in the sympathetic ganglia

The dose (ED_{90}) of tropenziline required to produce a certain effect on tonicized choledochoduodenal junction was four times the dose of atropine The action of tropenziline on other organs, too, has been reported to be weaker than that of atropine (TAESCHLER et al 1960, LECCHINI et al 1969) In their in vivo experiments, TAESCHLER et al (1960) found that the dose of tropenziline per weight unit had to be about five times that of atropine to produce same effect on the activity of the cat's small intestine, and on the vagus stimulated small intestine of the cat and dog They reported that tropenziline acts more selectively than atropine on the visceral parasympathetic ganglia and especially by this route spasmolytically

Papaverine, the agent acting directly on the smooth muscle cell, had a weaker action than the anticholinergic agents on the neurogenic spasm of the rabbit's choledochoduodenal junction Its effect, moreover, was of short duration Papaverine has been found to reduce the release of acetylcholine from guinea pig intestine (IDANPAAN HEIKKILA 1968), which may perhaps play a role in the spasmolysis produced by papaverine

The results revealed that, of the drugs studied, oxyphencyclimine and atropine were the best relaxants of the neurogenic spasm of rabbit's choledochoduodenal junction The oxyphencyclimine action was of longer duration than that of atropine Compared with N-butyl hyoscine, tiemonium and tropenziline, the antispasmodic action of these anticholinergic agents was better whereas they did not appreciably affect the blood pressure Papaverine produced only a transient relaxation of the neurogenic spasm of the choledochoduodenal junction

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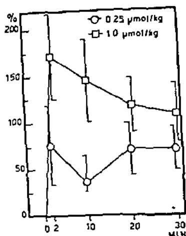


Fig 12

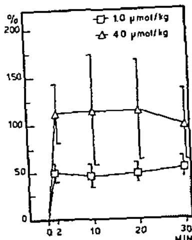


Fig 13

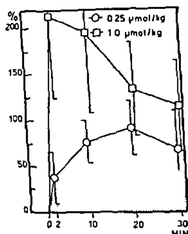


Fig 14

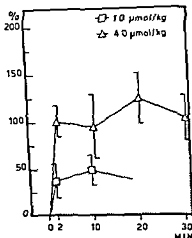


Fig 15

Increase of the choledochoduodenal flow in rabbits after the administration of atropine (Fig 12), N butyl hyoscine (Fig 13), tiemonium (Fig 14) and oxyphencyclimine (Fig 15) given 5 min after barium (chloride) 4 0 μmol/kg. The flow during one minute was measured at given times and the increase in flow was calculated as a percentage of the decrease in flow due to barium (chloride), recorded before the administration of spasmolytic Shaded area respective increase in flow without spasmolytic Means ± S E M are given. 5—6 animals in each group

An oxyphencyclimine (Fig 15) dose of 1 0 μmol/kg had no significant action on the rate of perfusion After a dose of 4 0 μmol/kg the flow in the 2nd minute showed a rate increased by 101 per cent ($p<0\ 001$) and even after 30 minutes, still by 104 per cent ($p<0\ 01$)

VII Effect of spasmolytics on the myogenic spasm of the choledochoduodenal junction

Studies were also carried out concerning the ability of spasmolytics to relax a myogenic spasm, induced by barium chloride, of the choledocho duodenal junction The doses of spasmolytics used were such as to increase the choledochoduodenal flow which had slowed down after barium chloride administration The spasmolytic under examination was adminis tered 5 minutes after the spasmogenic agent (4 0 μmol barium++ per kg body weight), by which time the choledochoduodenal flow had fallen by the average of 57—69 per cent The spasmolytics tested were the same as in the preceding section

RESULTS

Spasmolytic induced increase in the choledochoduodenal flow inhibited by barium chloride

The results (increase in flow) are given as a percentage of decrease in flow due to barium chloride and recorded before the administration of spasmolytic

In the anaesthetized experimental animal the increase in flow recorded two minutes after an atropine (Fig 12) dose of 0 25 μmol/kg was 76 per cent ($p<0\ 05$) Later during the experiment the action of this dose was no longer significant After a dose of 1 0 μmol/kg, perfusion first changed vigorously, the increase in flow after 2 minutes being 171 per cent ($p<0\ 001$) and even after 30 minutes 108 per cent ($p<0\ 05$)

With the N butyl hyoscine (Fig 13) dose of 1 0 μmol/kg the flow increase observed after 2 minutes was 50 per cent ($p<0\ 001$) Two and 10 minutes after a fourfold dose the flow increases were 112 ($p<0\ 001$) and 115 per cent ($p<0\ 05$) respectively but subsequently the observations did not significantly differ from control values

Increases in flow recorded two, 10 and 20 minutes after a tiemonium dose of 0 25 μmol/kg (Fig 14) were 39, 77 ($p<0\ 05$) and 92 per cent ($p<0\ 05$) respectively A four times larger dose modified the perfusion so that the increase after two minutes was 213 per cent ($p<0\ 01$) By the end of 20 minutes the average increase was 134 per cent ($p<0\ 05$)

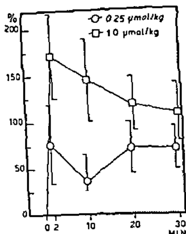


Fig 12

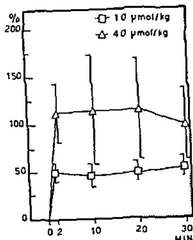


Fig 13

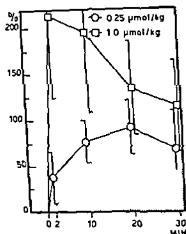


Fig 14

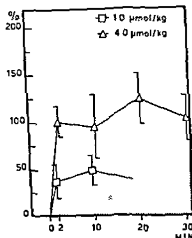


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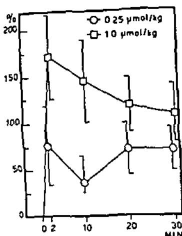


Fig 12

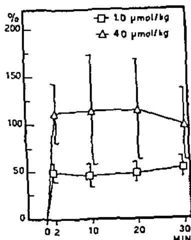


Fig 13

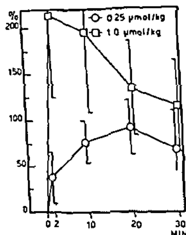


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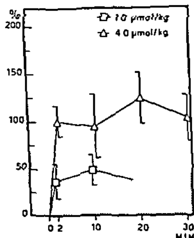


Fig 15

Increase of the choledochoduodenal flow in rabbits after the administration of atropine (Fig 12), N butyl hyoscine (Fig 13), tiemonium (Fig 14) and oxyphencyclimine (Fig 15) given 5 min after barium (chloride) 4 0 µmol/kg The flow during one minute was measured at given times and the increase in flow was calculated as a percentage of the decrease in flow due to barium (chloride), recorded before the administration of spasmolytic Shaded area respective increase in flow without spasmolytic. Means ± S E M are given 5—6 animals in each group

An oxyphencyclimine (Fig 15) dose of 1 0 µmol/kg had no significant action on the rate of perfusion After a dose of 4 0 µmol/kg the flow in the 2nd minute showed a rate increased by 101 per cent ($p < 0\,001$) and even after 30 minutes, still by 104 per cent ($p < 0\,01$)

VII Effect of spasmolytics on the myogenic spasm of the choledochoduodenal junction

Studies were also carried out concerning the ability of spasmolytics to relax a myogenic spasm, induced by barium chloride, of the choledocho duodenal junction. The doses of spasmolytics used were such as to increase the choledochoduodenal flow which had slowed down after barium chloride administration. The spasmolytic under examination was adminis tered 5 minutes after the spasmogenic agent (4.0 μmol barium++ per kg body weight), by which time the choledochoduodenal flow had fallen by the average of 57—69 per cent. The spasmolytics tested were the same as in the preceding section.

RESULTS

Spasmolytic-induced increase in the choledochoduodenal flow inhibited by barium chloride

The results (increase in flow) are given as a percentage of decrease in flow due to barium chloride and recorded before the administration of spasmolytic.

In the anaesthetized experimental animal the increase in flow recorded two minutes after an atropine (Fig. 12) dose of 0.25 μmol/kg was 76 per cent ($p<0.05$). Later during the experiment the action of this dose was no longer significant. After a dose of 1.0 μmol/kg, perfusion first changed vigorously, the increase in flow after 2 minutes being 171 per cent ($p<0.001$) and even after 30 minutes 108 per cent ($p<0.05$).

With the N butyl hyoscine (Fig. 13) dose of 1.0 μmol/kg the flow increase observed after 2 minutes was 50 per cent ($p<0.001$). Two and 10 minutes after a fourfold dose the flow increases were 112 ($p<0.001$) and 115 per cent ($p<0.05$) respectively but subsequently the observations did not significantly differ from control values.

Increases in flow recorded two, 10 and 20 minutes after a tiemonium dose of 0.25 μmol/kg (Fig. 14) were 39, 77 ($p<0.05$) and 92 per cent ($p<0.05$) respectively. A four times larger dose modified the perfusion so that the increase after two minutes was 213 per cent ($p<0.01$). By the end of 20 minutes the average increase was 134 per cent ($p<0.05$).

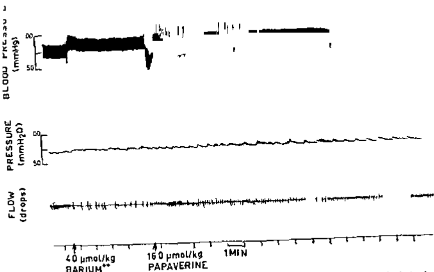


Fig 17 Effect of papaverine given 5 min after barium (chloride) on the choledocho duodenal flow common duct pressure and blood pressure in a rabbit

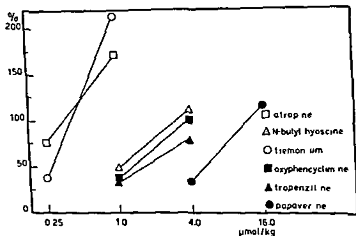


Fig 18 Effect of spasmolytics on the choledochoduodenal flow inhibited by barium (chloride) Log dose/response lines The flow was measured during the 2nd minute after the administration of each spasmolytic Increase in flow recorded *after each* spasmolytic was compared with the decrease in flow due to barium (chloride) observed before the administration of the spasmolytic

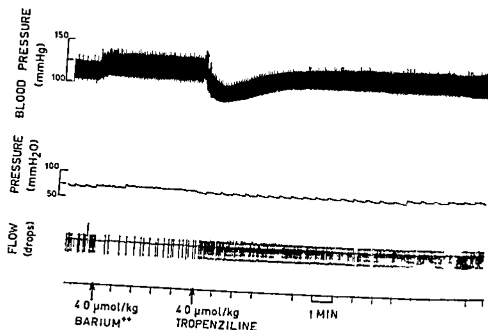
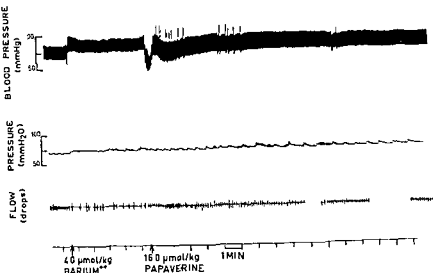


Fig 16 Effect of tropenziline given 5 min after barium (chloride) on the choledochoduodenal flow common duct pressure and blood pressure in a rabbit

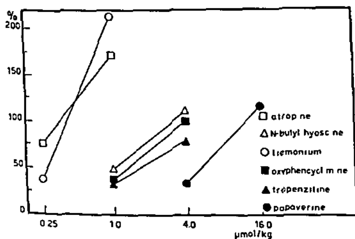
A tropenziline dose of 1 0 µmol/kg changed the flow so that the increase recorded after two minutes was 33 per cent ($p<0\ 01$), while subsequently the action was no longer significant With a dose of 4 0 µmol/kg (Fig 16) the increase in flow recorded after two minutes was 79 per cent ($p<0\ 001$) and after 30 minutes it was even 105 per cent ($p<0\ 05$)

With papaverine, a dose of 4 0 µmol/kg had no significant action on the choledochoduodenal junction A four times larger dose (Fig 17) produced an increase of short duration in the choledochoduodenal flow Flow change recorded after two minutes was 116 per cent ($p<0\ 001$) but later observations did not significantly differ from control values

The action of most spasmolytic doses was at its maximum in the 2nd minute after administration For comparing the potencies of spasmolytics at that time log dose/response lines were drawn for each spasmolytic (Fig 18) ED $_{90}$ dose of spasmolytics, determined in same way as in the preceding section, were atropine 0 31, N butyl hyoscine 2 43, tiemonium 0 38, oxyphencyclimine 3 15, tropenziline 5 40 and papaverine 10 30 µmol/kg



Fg 17 Effect of papaverine given 5 min after barium (chloride) on the choledocho duodenal flow common duct pressure and blood pressure in a rabbit



Fg 18 Effect of spasmolytics on the choledochoduodenal flow inh bited by barium (chloride) Log dose/response l nes The flow was measured during the 2nd m nute after the administration of each spasmolytic Increase in flow recorded after each spasmolyt c was compared with the decrease in flow due to barium (chloride) observed before the adm nistration of the spasmolytic

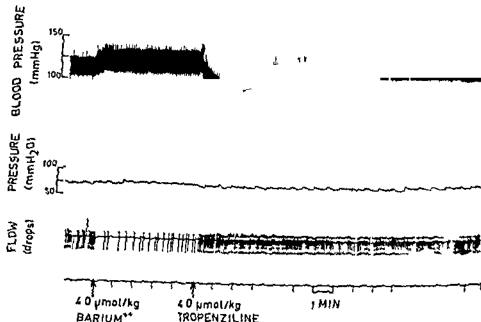


Fig 16 Effect of tropenziline given 5 min after barium (chloride) on the choledochoduodenal flow common duct pressure and blood pressure in a rabbit

A tropenziline dose of 1 0 μmol/kg changed the flow so that the increase recorded after two minutes was 33 per cent ($p<0\ 01$), while subsequently the action was no longer significant With a dose of 4 0 μmol/kg (Fig 16) the increase in flow recorded after two minutes was 79 per cent ($p<0\ 001$) and after 30 minutes it was even 105 per cent ($p<0\ 05$)

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The action of most spasmolytic doses was at its maximum in the 2nd minute after administration For comparing the potencies of spasmolytics at that time log dose/response lines were drawn for each spasmolytic (Fig 18) ED_{50} dose of spasmolytics, determined in same way as in the preceding section, were atropine 0 31, N-butyl hyoscine 2 43, tiemonium 0 38, oxyphencyclimine 3 15, tropenziline 5 40 and papaverine 10 30 μmol/kg

DISCUSSION

The barium ion acts directly on the smooth muscle and also stimulates nervous structures (see p. 18). In the present study, atropine and tiemonium were found to relax the spasm, induced by barium chloride in the choledochoduodenal junction, better than the other spasmolytics under examination. In other organs also, such as e.g. the rat's isolated ileum (LULLMANN 1950) an the dog's intestine (NECHELES et al. 1953), atropine has been found to be antagonistic to barium. Tiemonium has both a musculotropic action like that of papaverine, and an anticholinergic action especially on the intramural ganglia (see LAB. DE RECH. MAYVERNAY 1962). The spasmolysis induced by atropine and tiemonium probably results from their musculotropic action and their anticholinergic action on the nervous structures stimulated by barium ion.

As for the other anticholinergic agents, the doses required to produce an anti barium action on the choledochuodenal junction were considerably larger. The relaxing effect of these agents is probably mainly due to their anticholinergic action on nervous structures. Oxyphencyclimine had a good anti neostigmine and a weak anti barium action, and a slight effect on the blood pressure after neostigmine administration. All this suggests that the spasmolytic action recorded may be mainly based on the anticholinergic action in the peripheral nerve ending. Tropenziline administered after barium chloride reduced the blood pressure substantially, which may have been due to its strong inhibiting action on ganglia (TAESCHLER et al. 1960).

Papaverine relaxed the myogenic spasm of the choledochoduodenal junction relatively better than the neurogenic spasm. Compared with the anticholinergic agents, its action on the spasm induced by barium chloride was also of short duration.

Changes in the arterial blood pressure

Barium chloride (4.0 µmol barium++ per kg body weight) produced an average blood pressure rise of 23 mmHg. The initial level was restored in about 15 minutes.

The blood pressure changes produced by spasmolytics administered after the barium chloride are presented in Table 4.

After an atropine dose of 1.0 µmol/kg the maximum blood pressure fall was 18 mmHg.

N-butyl hyoscine doses of 1.0 and 4.0 µmol/kg produced a transient fall in blood pressure which amounted to 15 mm Hg and 21 mmHg, respectively.

Tiemonium, in a dose of 1.0 µmol/kg, reduced the blood pressure by an average of 10 mmHg.

Oxyphencyclimine, in a dose of 4.0 µmol/kg, reduced the blood pressure by 15 mmHg.

Tropenziline, with the doses used, reduced the blood pressure considerably. 1.0 µmol/kg produced an average fall of 26 mmHg, and after the dose of 4.0 µmol/kg the maximum blood pressure fall averaged 39 mmHg.

Papaverine, in a dose of 4.0 µmol/kg, produced an average blood pressure fall of 15 mmHg and the dose of 16 µmol/kg one of 35 mmHg.

Table 4

Changes in the rabbit blood pressure after administration of various spasmolytics
Spasmolytics were given 5 min after barium (chloride) 4.0 µmol/kg

Drug		Dose µmol/kg	Mean decrease in blood pressure (mmHg±S.E.M.)		
			0.5 min	2 min	10 min
Atropine	(6)	0.25	2±1	5±2	6±3
"	(5)	1.0	16±4 ***	18±4 **	14±2
N-butyl hyoscine	(5)	1.0	15±2 ***	13±2 *	6±3
"	(5)	4.0	21±4 ***	17±2 **	8±2
Tiemonium	(5)	0.25	3±1	8±2	12±3
"	(5)	1.0	10±2 **	11±2	12±3
Oxyphencyclimine	(5)	1.0	1±1	7±1	10±3
"	(5)	4.0	13±2 ***	15±3 **	13±4
Tropenziline	(5)	1.0	26±2 ***	20±3 **	9±3
"	(5)	4.0	30±10 **	39±9 **	6±3
Papaverine	(5)	4.0	15±2 ***	8±1	8±2
"	(5)	16.0	35±5 ***	17±2 **	11±5

Number of animals in brackets. Statistical significances were calculated by comparing the changes in blood pressure observed after spasmolytics with the respective changes after barium (chloride) alone

* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

DISCUSSION

The barium ion acts directly on the smooth muscle and also stimulates nervous structures (see p 18) In the present study, atropine and tiemonium were found to relax the spasm, induced by barium chloride in the choledochoduodenal junction, better than the other spasmolytics under examination In other organs also, such as e g the rat's isolated ileum (LULLMANN 1950) an the dog's intestine (NECHELES et al 1953), atropine has been found to be antagonistic to barium Tiemonium has both a musculotropic action like that of papaverine, and an anticholinergic action especially on the intramural ganglia (see LAB DE RECH MAYVERNAY 1962) The spasmolysis induced by atropine and tiemonium probably results from their musculotropic action and their anticholinergic action on the nervous structures stimulated by barium ion

As for the other anticholinergic agents, the doses required to produce an anti barium action on the choledochuodenal junction were considerably larger The relaxing effect of these agents is probably mainly due to their anticholinergic action on nervous structures Oxyphencyclimine had a good anti neostigmine and a weak anti barium action, and a slight effect on the blood pressure after neostigmine administration All this suggests that the spasmolytic action recorded may be mainly based on the anticholinergic action in the peripheral nerve ending Tropenziline adm nistered after barium chloride reduced the blood pressure substantially, which may have been due to its strong inhibiting action on ganglia (TAESCHLER et al 1960)

Papaverine relaxed the myogenic spasm of the choledochoduodenal junction relatively better than the neurogenic spasm Compared with the anticholinergic agents, its action on the spasm induced by barium chloride was also of short duration

Changes in the arterial blood pressure

Barium chloride (4 0 µmol barium++ per kg body weight) produced an average blood pressure rise of 23 *mmHg* *The initial* level was restored in about 15 minutes

The blood pressure changes produced by spasmolytics administered after the barium chloride are presented in Table 4

After an atropine dose of 1 0 µmol/kg the maximum blood pressure *fall was 18* mmHg

N-butyl hyoscine doses of 1 0 and 4 0 µmol/kg produced a transient fall in blood pressure which amounted to 15 mm Hg and 21 mmHg, respectively

Tiemonium, in a dose of 1 0 µmol/kg, reduced the blood pressure by an average of *10* mmHg

Oxyphencyclimine, in a dose of 4 0 µmol/kg, reduced the blood pressure *by 15* mmHg

Tropenziline, with the doses used, reduced the blood pressure considerably 1 0 µmol/kg produced an average fall of 26 mmHg, and after the dose of 4 0 µmol/kg the maximum blood pressure fall averaged 39 mmHg

Papaverine, in a dose of 4 0 µmol/kg, produced an average blood pressure fall of 15 mmHg and the dose of 16 µmol/kg one of 35 mmHg

Table 4

Changes in the rabbit blood pressure after administration of various spasmolytics Spasmolytics were given 5 min after barium (chloride) 4 0 µmol/kg

Drug		Dose µmol/kg	Mean decrease in blood pressure (mmHg±S E M)		
			0 5 min	2 min	10 min
Atropine	(6)	0 25	2±1	5±2	6±3
»	(5)	1 0	16±4 ***	18±4 **	14±2
N butyl hyoscine	(5)	1 0	15±2 ***	13±2 *	6±3
»	(5)	4 0	21±4 ***	17±2 **	8±2
Tiemonium	(5)	0 25	3±1	8±2	12±3
»	(5)	1 0	10±2 **	11±2	12±3
Oxyphencyclimine	(5)	1 0	1±1	7±1	10±3
»	(5)	4 0	13±2 ***	15±3 **	13±4
Tropenziline	(5)	1 0	26±2 ***	20±3 **	9±3
»	(5)	4 0	30±10 **	39±9 **	6±3
Papaverine	(5)	4 0	15±2 ***	8±1	8±2
»	(5)	16 0	35±5 ***	17±2 **	11±5

Number of animals in brackets Statistical significances were calculated by comparing the changes in blood pressure observed after spasmolytics with the respective changes after barium (chloride) alone

* = $p<0\ 05$ ** = $p<0\ 01$, *** = $p<0\ 001$

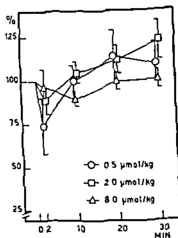


Fig 19

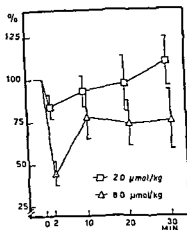


Fig 20

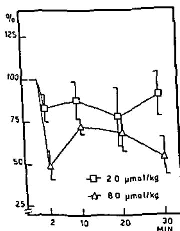


Fig 21

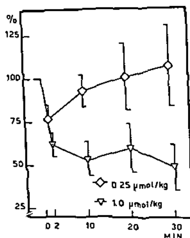


Fig 22

Choledochoduodenal flow in rabbits after the administration of morphine (Fig 19), pethidine (Fig 20) pentazocine (Fig 21) and phenazocine (Fig 22) as a percentage of the initial flow (mean/minute in 5 pre injection minutes) The post injection flow during one minute was measured at given times Means ± S E M are given 5—6 animals *in each group*

perfusion so that, in the 2nd minute, it was 23 per cent below the initial level ($p < 0.05$) After a dose four times larger the rate of perfusion remained reduced throughout the period of observation and was 38—51 per cent ($p < 0.01$) below the initial level

VIII Effect of analgesics and related drugs on the choledochoduodenal junction and the common bile duct pressure

In order to study the effects of analgesics and related drugs on rabbit biliary tract the changes they produced in the tone of the choledochoduodenal junction and in the common bile duct pressure were measured The following agents were used for this study morphine, pethidine, pentazocine, phenazocine, dextropropoxyphene and dextromethorphan Blood pressure was recorded in the course of the experiments

RESULTS

Changes in the choledochoduodenal flow

The morphine action (Fig 19) was examined using doses of 0 5, 2 0 and 8 0 μmol/kg Preliminary tests showed that morphine in doses below 0 5 μmol/kg neither raised nor lowered the tone of the choledochoduodenal junction or blood pressure Nor did any of the doses used produce a statistically significant change in the rate of flow

Pethidine (Fig 20) in a dose of 2 0 μmol/kg produced no change of statistical significance in the flow With the dose of 8 0 μmol/kg, the rate of flow in the 2nd minute was 55 per cent (p<0 001) lower than the initial rate Subsequently during the experiment it was an average of 26—22 per cent below the initial value, but the difference was not significant

The action of pentazocine (Fig 21) on the choledochoduodenal junction was also examined with doses of 2 0 and 8 0 μmol/kg With the smaller dose, the rate of perfusion remained throughout the experiment below the initial level, even though the difference was not significant The dose of 8 0 μmol/kg was found to have a marked inhibiting action on choledochoduodenal flow Average perfusion was reduced in two minutes by 51 per cent (p<0 001) Subsequently the rate of perfusion at the moments of observation was 27—45 per cent (p<0 01) below the initial level

Phenazocine (Fig 22) in a dose of 0 25 μmol/kg slowed the rate of

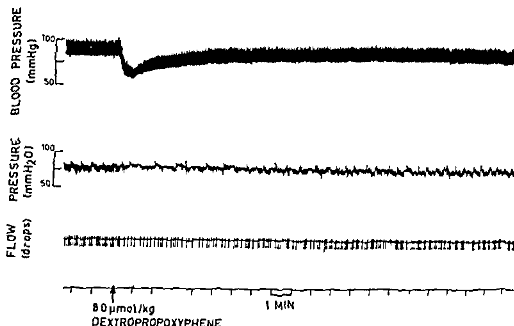


Fig 23 Effect of dextropropoxyphene on the choledochoduodenal flow, common duct pressure and blood pressure in a rabbit

Dextropropoxyphene produced no significant change in the choledochoduodenal flow with a dose of 2 0 μmol/kg After a dose of 8 0 μmol/kg (Fig 23) the flow in the 2nd minute was an average of 36 per cent ($p<0\ 05$) below the initial level to which it was restored in 10 minutes

Dextromethorphan also decreased the rate of the choledochoduodenal flow After a dose of 2 0 μmol/kg the flow in the 2nd minute was 32 per cent ($p<0\ 001$) and in the 10th minute 30 per cent ($p<0\ 05$) below the initial level With a fourfold dose (Fig 24) the rate of perfusion at first fell markedly In the 2nd minute the flow was 54 per cent ($p<0\ 001$) below the initial level, but subsequently the changes were not significant

Changes in the common bile duct pressure

When the action of drugs on the common bile duct pressure was examined the pressure was recorded for a minimum of 30 minutes after drug administration The maximum rise was obtained within this period

The present results concerning the action of drugs on the rabbit's common bile duct pressure are given in Table 5 It shows the changes produced

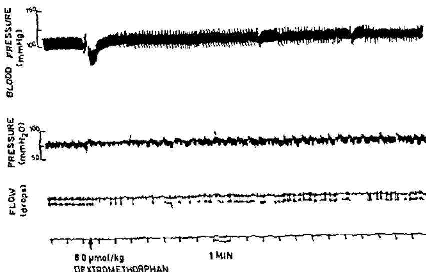


Fig. 24 Effect of dextromethorphan on the choledochoduodenal flow, common duct pressure and blood pressure in a rabbit

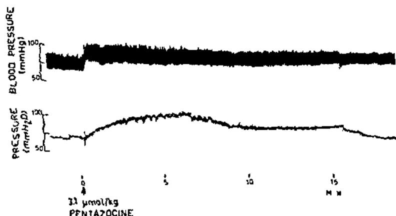


Fig 25 Effect of pentazocine on the common duct pressure and blood pressure in a rabbit.

by the doses of the greatest efficacy. The drugs examined had only a slight effect in the conditions used for the experiments Pentazocine (Fig 25), pethidine and phenazocine produced the greatest average increases.

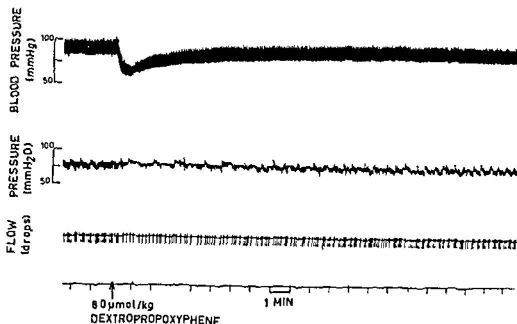


Fig 23 Effect of dextropropoxyphene on the choledochoduodenal flow, common duc pressure and blood pressure in a rabbit

Dextropropoxyphene produced no significant change in the choledochoduodenal flow with a dose of 2 0 µmol/kg After a dose of 8 0 µmol/kg (Fig 23) the flow in the 2nd minute was an average of 36 per cent ($p<0\ 05$) below the initial level to which it was restored in 10 minutes

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Changes in the common bile duct pressure

When the action of drugs on the common bile duct pressure was examined the pressure was recorded for a minimum of 30 minutes after drug administration The maximum rise was obtained within this period

The present results concerning the action of drugs on the rabbit's common bile duct pressure are given in Table 5 It shows the changes produced

Pentazocine dose 2 0 μmol/kg, produced a distinct rise in blood pressure by up to 18 mmHg, lasting throughout the experiment The dose of 8 0 μmol/kg first produced a pronounced blood pressure fall of 35 mmHg, after which the blood pressure rose so as to exceed the initial level

Phenazocine in a dose of 1 0 μmol/kg reduced the blood pressure transiently by 10 mmHg

Dextropropoxyphene, with the doses used, gave the most pronounced fall in blood pressure The doses of 2 0 and 8 0 μmol/kg at first reduced the blood pressure by 16 and 51 mmHg, respectively With the larger dose, a somewhat reduced level persisted up to the 10th minute

Dextromethorphan, 2 0 μmol/kg, had a raising action on blood pressure A fourfold dose reduced the blood pressure for a short period by 33 mmHg

DISCUSSION

The effects of analgesics on the intestine differ from one species to another In man, morphine inhibits the peristalsis, besides having a spasmogenic action (Daniel & Bogoch 1958) Morphine probably affects the intestinal peristalsis and tone through different mechanisms, since the guinea pig and rabbit only show the inhibition of peristalsis but no spasmogenic action on the intestine (Schaumann 1954, Daniel & Bogoch 1958) Nor did the present study reveal any spasmogenic action of morphine on the rabbit s choledochoduodenal junction This might be because morphine may reduce the release of transmitter substances from the intestine (Paton 1957, Schaumann 1957, Cox & Weinstock 1966, Trendelenburg 1957, Cairnie al 1961, Szerb 1961) and act directly on the intestinal smooth muscle (Daniel 1964)

Pethidine, despite its anticholinergic activity, may produce a spasm of both the intestinal and bronchial smooth muscle (Murphree 1965) In the rabbit the choledochoduodenal junction was found to contract after a pethidine dose of 8 0 μmol/kg

Pentazocine retards the emptying of the human stomach and reduces intestinal motility, and it has also been reported to produce a contraction of the sphincter of Oddi (Hinshaw et al 1966, Danhof 1967) A pentazocine dose of 2 0 μmol/kg produced in the rabbit a blood pressure rise of long duration When the dose of 8 0 μmol/kg was used, the choledochoduodenal junction was found to contract There was also a transient fall in the blood pressure which later rose so as to exceed the initial level The decrease in flow produced by pentazocine may perhaps be due to sympathicus stimulation and the subsequent constriction of the sphincter of papillae, since pentazocine had a prolonged blood pressure raising effect

Table 5

Effect of analgesics and related drugs on the common bile duct pressure in rabbits

Drug		Dose µmol/kg	Common bile duct pressure (mmH_2O)		
			mean initial	mean of maximal increases	range of increases
Morphine	(6)	2 0	62	11	0—30
Pethidine	(6)	8 0	60	18	13—35
Pentazocine	(6)	3 1	79	23	13—37
Phenazocine	(6)	1 0	83	18	14—24
Dextropropoxyphene	(5)	2 7	77	8	0—16
Dextromethorphan	(5)	5 7	53	9	7—12

Number of animals in brackets

Changes in the arterial blood pressure

The blood pressure changes produced by analgesics and related drugs are presented in Table 6

After morphine doses of 2 0 and 8 0 µmol/kg the blood pressure remained slightly but significantly below its initial level throughout the experiment

Pethidine, in a dose of 8 0 µmol/ kg, produced a transient fall of 19 mmHg.

Table 6

Effect of analgesics and related drugs on rabbit blood pressure

Drug		Dose µmol/kg	Mean change in blood pressure (mmHg ± S E M)			
			0 5 min	2 min	10 min	30 min
Morphine	(6)	0 5	— 3 ± 2	— 3 ± 2	+ 3 ± 2	+ 3 ± 2
»	(12)	2 0	— 6 ± 2 **	—10 ± 3 *	— 9 ± 1 ***	— 5 ± 2 *
»	(11)	8 0	— 6 ± 1 ***	—10 ± 1 ***	—11 ± 1 ***	— 8 ± 1 ***
Pethidine	(10)	2 0	— 4 ± 2	— 4 ± 2	— 3 ± 3	— 3 ± 2
»	(13)	8 0	—19 ± 3 ***	—12 ± 4 **	— 7 ± 4	— 6 ± 3
Pentazocine	(7)	2 0	+16 ± 4 **	+18 ± 4 ***	+14 ± 3 **	+17 ± 6 *
»	(5)	8 0	—35 ± 4 ***	+13 ± 5 *	+10 ± 5	+ 7 ± 6
Phenazocine	(10)	0 25	± 0 ± 1	— 1 ± 2	— 3 ± 2	— 3 ± 2
»	(11)	1 0	—10 ± 3 **	+ 3 ± 3	— 6 ± 3	— 4 ± 3
Dextropropoxyphene	(9)	2 0	—16 ± 3 ***	— 7 ± 3 **	— 3 ± 2	— 3 ± 2
»	(7)	8 0	—51 ± 8 ***	—36 ± 5 ***	— 7 ± 3 *	— 1 ± 2
Dextromethorphan	(10)	2 0	— 3 ± 3	+ 8 ± 2 **	+ 3 ± 2	+ 4 ± 1 *
»	(6)	8 0	—33 ± 3 ***	— 1 ± 5	+ 5 ± 3	+ 3 ± 4

Number of animals in brackets * = p<0 05, ** = p<0 01, *** = p<0 001

Pentazocine, *dose 2 0 µmol/kg*, produced a distinct rise in blood pressure *by up to 18 mmHg* lasting throughout the experiment The dose of 8 0 µmol/kg first produced a pronounced blood pressure fall of 35 mmHg, after which the blood pressure rose so as to exceed the initial level

Phenazocine in a dose of 1 0 µmol/kg reduced the blood pressure transiently by 10 mmHg

Dextropropoxyphene, with the doses used, gave the *most pronounced* fall in blood pressure The doses of 2 0 and 8 0 µmol/kg at first reduced the blood pressure by 16 and 51 mmHg, respectively With the *larger dose*, a somewhat reduced level persisted up to the 10th minute

Dextromethorphan 2 0 µmol/kg had a raising action on blood pressure A fourfold dose reduced the blood pressure for a short period by 33 mmHg

DISCUSSION

The effects of analgesics on the intestine differ from one species to another In *man, morphine* inhibits the peristalsis, besides having a spasmogenic action (DANIEL & BOGOCH 1958) Morphine probably affects the intestinal peristalsis and tone through different mechanisms, since the guinea pig and rabbit only show the inhibition of peristalsis but no spasmogenic action on the intestine (SCHAUMANN 1954, DANIEL & BOGOCH 1958) Nor did the present study reveal any spasmogenic action of morphine on the rabbit s choledochoduodenal junction This might be because morphine may reduce the release of transmitter substances from the intestine (PATON 1957, SCHAUMANN 1957, COX & WEINSTOCK 1966, TRENDELENBURG 1957, CAIRNIE al 1961, SZERB 1961) and act directly on the intestinal smooth muscle (DANIEL 1964)

Pethidine, despite its anticholinergic activity, may produce a spasm of both the intestinal and bronchial smooth muscle (MURPHREE 1965) In the rabbit, the choledochoduodenal junction was found to contract after a pethidine dose of 8 0 µmol/kg

Pentazocine retards the emptying of the human stomach and reduces intestinal motility, and it has also been reported to produce a contraction of the sphincter of Oddi (HINSHAW et al 1966, DANHOF 1967) A pentazocine dose of 2 0 µmol/kg produced in the rabbit a blood pressure rise of *long duration* When the dose of 8 0 µmol/kg was used, the choledochoduodenal junction was *found to contract* There was also a transient fall in the blood pressure which later rose so as to *exceed the initial level* The decrease in flow produced by pentazocine may perhaps be *due to sympathicus* stimulation and the subsequent constriction of the sphincter of papillae since pentazocine had a prolonged blood pressure raising effect

Pentazocine has been reported to raise the content of catecholamines in the human blood (TAMMISTO et al 1970)

Phenazocine is a benzomorphan derivative closely related to pentazocine Its analgesic dose in man is less than one tenth of that of pentazocine Phenazocine also caused a contraction of the rabbit's choledochoduodenal junction, a finding probably associated with its retarding effect on the motility of the gastrointestinal tract (JAFFE 1965)

Dextropropoxyphene is an agent structurally related to methadone, and has the same effects on the gastrointestinal tract as codeine though their incidence is somewhat lower (JAFFE 1965) In the present study, dextropropoxyphene in a dose of 8 0 μmol/kg produced a very marked blood pressure fall, compared with the other drugs The fall was less transient than that following equal doses of pentazocine and dextromethorphan The spasm of the choledochoduodenal junction due to the effect of dextropropoxyphene may be connected with the blood pressure fall it produced (see p 18)

Dextromethorphan, an antitussive structurally near to codeine, has no analgesic properties and rarely produces gastrointestinal disturbances (JAFFE 1965) In the present study it inhibited choledochoduodenal flow and also raised blood pressure The decrease in choledochoduodenal flow may be due to the sympathicus stimulation and subsequent constriction of the sphincter of papillae

With the present arrangement of experiments, the action of drugs on the common bile duct pressure was slight The function of the gallbladder as a pressure regulator and the reduced bile secretion due to the starvation of the animals may have contributed to the slight responses recorded

IX General discussion

The present study of the effects of drugs on the rabbit's choledochoduodenal junction used a method similar in theory to the one used by e g CURRERI & GALE (1950), ERDMANN & HENNE (1953), and ROBELET et al (1965) This method demonstrates drug effects more sensitively than does the measurement of the common bile duct pressure (ERDMANN & HENNE 1953) On the average, three experiments per animal were carried out In order to eliminate the interactions of various agents, each experiment was carried out after the effects of the previously administered drug were no longer noticeable The experimental animals were anaesthetized with pentobarbital, and the action of pentobarbital on the choledochoduodenal flow was also determined under light ether anaesthesia It was not found to affect the choledochoduodenal flow significantly Nor did an additional dose of pentobarbital administered during the course of pentobarbital anaesthesia significantly change the tone of the choledochoduodenal junction

The blood pressure of the animals was monitored in order to assess the tolerance and circulatory effects of drugs LUETH (1931) found that the tone of the choledochoduodenal junction increased in connection with a heavy fall in blood pressure, a finding he attributed to anoxia Bearing this in mind efforts were made to use drugs with a not too potent reducing action on blood pressure

The choledochoduodenal flow underwent little change due to drugs acting on the adrenergic system For adrenaline, noradrenaline and phentolamine the changes were not significant However, symptoms of a contraction of the choledochoduodenal junction were noted after noradrenaline administration, and symptoms of relaxation after a phentolamine dose of 0 25 mg/kg This seems to suggest that the rabbit's choledochoduodenal junction contains alpha receptors mediating contraction The transient increase in choledochoduodenal flow obtained with isoprenaline and the short term retardation in the flow after propranolol are probably associated, the former with a stimulation and the latter with an inhibition of the beta receptors of the choledochoduodenal junction

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Drug action on the neurogenic and myogenic spasm of the choledochoduodenal junction was studied using atropine, N-butyl hyoscine, tiemonium, oxyphencyclimine, tropenziline and papaverine Of these agents, N-butyl hyoscine, tropenziline and tiemonium have a strong anticholinergic activity in the ganglia and, in addition, tiemonium has a musculotropic action similar to that of papaverine (see LAB DE RECH MAYVERNAY 1962, EHRHART & RUSCHIG 1968)

According to some preliminary tests, the ability of the spasmolytics to relax a nontonicized choledochoduodenal junction was slight On the other hand, all the drugs studied relaxed its neurogenic and myogenic spasm The good antispasmodic effect recorded for atropine and oxyphencyclimine in relaxing the neurogenic spasm was probably due to their marked anticholinergic activity in the peripheral nerve ending since, when given after neostigmine, they had no significant action on the blood pressure Atropine, like tiemonium, was a good relaxant of the myogenic spasm, a finding to suggest that it also has a good myolytic effect The antispasmodic action of papaverine was of shorter duration than that of the anticholinergic agents as regards the relaxation of both the neurogenic and the myogenic spasm, but papaverine relaxed myogenic spasm relatively better than the other spasmolytic agents

The present studies of analgesics and related drugs, such as morphine, pethidine, pentazocine, dextropropoxyphene and dextromethorphan, revealed no correlation between their spasmogenic and known analgesic actions

The fact that morphine failed to contract the rabbit's choledochoduodenal junction is probably associated with morphine action on the release of transmitter substances and on the smooth muscle Morphine inhibited the contractions of guinea pig intestine, and this inhibition was correlated to the inhibition of acetylcholine release (SCHAUMANN 1957, PATON 1957, COX & WEINSTOCK 1966) Morphine has also been found to reduce the release of noradrenaline from the peripheral nerve ending (TRENDELENBURG 1957, CAIRNIE et al 1961)

Many drugs release in the organism biologically active substances which may affect the smooth muscle 5-hydroytryptamine (5-HT) stimulates the intestinal smooth muscle mainly via the nervous system, and also by a direct effect on the smooth muscle (DAY & VANE 1963) 5-HT, however, hardly participates in drug action on rabbit intestine According to ERSPAMER (1961) a minimum intravenous dose of 0 2 mg/kg 5-HT is required to produce a distinct effect on the rabbit intestine, and even a dose of 5 mg/kg only has an effect of very short duration In vitro, morphine inhibits the 5-HT action on guinea pig intestine (KOSTERLITZ & ROBINSON 1958, LEVIS 1960), where the nervous receptors are sensitive

to morphine (GADDUM & PICARELLI 1967, BARLOW & KHAN 1957, DAY & VANE 1963) On the other hand, 5-HT presumably plays a part in the spasmogenic effect of morphine and related drugs on the intestine of the dog (BURKS & LONG 1967)

Histamine may also affect the tone of the intestine In the guinea pig, it produces a contraction of the ileum by a direct action on muscle fibres (FELDBERG 1951, KOSTERLITZ & ROBINSON 1958, DAY & VANE 1963) Analgesics may cause a histamine release (FELDBERG & PATON 1951, SCHACHTER 1942, ZEPPA et al 1961), but histamine hardly plays any part in their intestinal action (DANIEL & BOGOCH 1958, BURGS & LONG 1967) Morphine reduces the response of guinea pig ileum to histamine (LEVIS 1960)

In the attempts to explain the action of analgesics and the related drugs on the intestine, attention has also been given to their anticholinesterase activity YOUNG et al (1955) and FOLDES et al (1959) claimed that anti cholinesterase activity could hardly be associated with the intestinal effects of morphine and related drugs, whereas EADIE et al (1948) assumed that cholinesterase inhibition may explain the peripheral side effects

Analgesics may perhaps also affect intestinal function by means of other mechanisms For example, they reduce the inhibitory effect of certain kinins on the rabbit intestine (BAUER et al 1966) Morphine may exert an inhibitory action on the intestine also through centrally mediated mechanisms (GREEN 1959, MARGOLIN 1963)

Besides contracting the choledochoduodenal junction, a pethidine dose of 8 0 μmol/kg also produced a moderate fall in blood pressure Pentazocine and dextromethorphan with the same dose produced a somewhat greater fall but of short duration With pentazocine, the blood pressure later rose so as to exceed the initial level Dextropropoxyphene reduced the blood pressure very heavily It is very probable that this heavy fall in blood pressure following the administration of dextropropoxyphene may play a part in the contraction that the drug produced in the choledochoduodenal junction

Drug action on the neurogenic and myogenic spasm of the choledochoduodenal junction was studied using atropine, N-butyl hyoscine, tiemonium, oxyphencyclimine, tropenziline and papaverine Of these agents, N-butyl hyoscine, tropenziline and tiemonium have a strong anticholinergic activity in the ganglia and, in addition, tiemonium has a musculotropic action similar to that of papaverine (see Lab de Rech Mayvernay 1962, Ehrhart & Ruschig 1968)

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X Summary

Drug action on the choledochoduodenal junction, on the neurogenic and myogenic spasm of the choledochoduodenal junction, on the common bile duct pressure and blood pressure in the rabbit anaesthetized with pentobarbital was studied Drug action on the tone of the choledochoduodenal junction was assessed by examining the changes in the rate of flow when physiological saline solution was perfused from the common bile duct to the duodenum The changes produced by drugs in the common bile duct pressure and blood pressure were measured electromanometrically

In studies with three catecholamines, noradrenaline, adrenaline, and isoprenaline, only isoprenaline was found to produce a significant change, i e a relaxation in the choledochoduodenal junction An alpha adrenergic blocking agent, phentolamine, did not significantly affect the choledochoduodenal junction, whereas a beta adrenergic blocking agent, propranolol, produced in it a slight, transient contraction A propranolol dose of 0 25 mg/kg raised the blood pressure transiently, while 1 0 mg/kg produced a blood pressure fall of long duration

The studied spasmolytic drugs, atropine, N-butyl hyoscine, tiemonium, oxyphencyclimine, tropenziline and papaverine, relaxed both the neurogenic spasm induced by neostigmine and the myogenic spasm induced by barium chloride in the rabbit's choledochoduodenal junction

The above-mentioned spasmolytic drugs were compared according to their efficacy in increasing the choledochoduodenal flow inhibited by neostigmine The order of their efficacy assessed on the basis of the approximate ED_{90} dose (in brackets µmol/kg) was the following atropine (0 11) >oxyphencyclimine (0 12)>N butyl hyoscine (0 20)>tropenziline (0 44) >tiemonium (0 75)>papaverine (15 60)

The order of efficacy of the above drugs, similarly assessed, in increasing the choledochoduodenal flow inhibited by barium chloride was atropine (0 31)>tiemonium (0 38)>N-butyl hyoscine (2 43)>oxyphencyclimine (3 15) >tropenziline (5 40)>papaverine (10 30)

Tropenziline, N butyl hyoscine and tiemonium, administered after neostigmine, reduced the blood pressure most After barium chloride administration, the heaviest fall in blood pressure was produced by tropenziline

The analgesics, pethidine, pentazocine, phenazocine and dextropropoxyphene as well as the antitussive dextromethorphan contracted the choledochoduodenal junction in the rabbit Morphine did not significantly affect its tone

The rise produced by analgesics and dextromethorphan in the common bile duct pressure in the rabbit anaesthetized with pentobarbital was slight, maximum 37 mmH_2O The greatest average rises were produced by pentazocine, pethidine and phenazocine

In studies with dextromethorphan and analgesics, pentazocine and dextromethorphan were found to raise the blood pressure Dextropropoxyphene produced the heaviest fall in blood pressure

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XII References

Ahlquist R. P, R A Huggins & R A Woodburg The pharmacology of benzylimidazoline (Priscol) *J Pharmacol exp Ther* 1947, 89, 271—288

Ambache N Interaction of drugs and the effect of cooling on the isolated mammalian intestine *J Physiol* (Lond.) 1946, 104, 266—287.

Ambache, N The nicotinic action of substances supposed to be purely smooth muscle stimulating (B) Effect of BaCl₂ and pilocarpine on the superior cervical ganglion *J Physiol* (Lond) 1949, 110, 64—172

Archibald E The experimental production of pancreatitis in animals as the result of the resistance of the common duct sphincter *Surg Gynec Obstet* 1919, 28, 529—545

Bainbridge, F A & H H Dale The contractile mechanism of the gall bladder and its extrinsic nervous control *J Physiol* (Lond) 1905—6, 33, 138—155

Barlow R B & I Khan The use of guinea pig ileum preparation for testing the activity of substances which imitate or antagonize the actions of 5 hydroxytryptamine and tryptamine *Brit J Pharmacol* 1959, 14, 553—558

Bauer G E Ziegler & H Konzett Zur Hemmwirkung von Kininen an isolierten Darmpreparaten *Naunyn-Schmiedebergs Arch exp Path Pharmak* 1966, 254, 235—244

Bayliss, W M & E H Starling The mechanism of pancreatic secretion *J Physiol* (Lond) 1902 28, 325—353

Beaver W T & W F Riker The quantitative evaluation of autonomic drugs on the isolated eye *J Pharmacol exp Ther* 1962 138, 48—56

Bensley B A Practical anatomy of the rabbit 8th ed revised by Craigie, E. H, Philadelphia The Blakiston Co, 1948

Benzi G F Berte A Crema & G M Frigo Actions of sympathomimetic drugs on the smooth muscle at the junction of the bile duct and duodenum studied in situ *Brit J Pharmacol* 1964 23, 101—114

Bergh G S The sphincter mechanism of the common bile duct in human subjects. *Surgery* 1942 11, 299—330

Bowman W C M J Rand & G B West Textbook of Pharmacology, Blackwell Scientific Publications, Oxford and Edinburgh 1968

Boyden E A Discussion *Minn Med* 1938 21, 877—880

Bulbring E Membrane potentials of smooth muscle fibres of the taenia coli of the guinea pig *J Physiol* (Lond.) 1954 125, 302—315

Bulbring E Correlation between membrane potential, spike discharge and tension in smooth muscle *J Physiol* (Lond) 1955 128, 200—221

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Doubilet, H & R. Colp Resistance of the sphincter of Oddi in the human. *Surg Gynec Obstet* 1937 **64**, 622—633

Doubilet, H. & J H Mulholland Recurrent acute pancreatitis Observations on etiology and surgical treatment *Ann Surg* 1948, **128**, 609—638

Doyon, M M Caractéres de la vesicule et du canal choledoque *Arch de Phys norm path* 1893 **5**, 710—719

Doyon M. M. De l action exercee par le systeme nerveux sur l appareil excreteur de la bile *Arch de Phys norm path* 1894, **6**, 19—31

Duchene-Marullaz, P., D Jovanovic N Busch & J Vacher Etude pharmacodynamique d un nouveau spasmolytique de synthese L iodure de α thienyl 1 phenyl 1 N methyl morpholinium 3 propanol 1 *Arch int. Pharmacodyn* 1963 **141**, 465—479

Eadie G S., F Bernheim & D B Fitzgerald The inhibition of the cholinesterase of rat brain by methadon *J Pharmacol exp Ther* 1948, **94**, 19—21

Edlund, T & A Lohi Are Ba ions a pure muscular stimulant of the rat s ileum but not of the guinea p g s? The species difference in spasmolytic potency of some ganglionic blocking agents. *Experientia* 1952, **8**, 156—157

Ehrhart G & H Ruschig Arzneimittel 1 Verlag Chemie GMBH, Weinheim/Bergstr 1968

Erdmann, W D & H F Henne Über spasmolytische Wirkungen am Sphincter Oddi. *Naunyn Schmiedebergs Arch exp Path Pharmak* 1953 **218**, 462—478

Erspamer V Recent research in the field of 5-hydroxytryptamine and related indolealkylamines. *Arzneimit el Forsch* 1961 **3**, 151—367

Felberg, W Effects of ganglion blocking substances on the small intestine. *J Physiol* (Lond.) 1951 **133**, 483—505

Feldberg W & D M Paton Release of histamine from skin and muscle in the cat by opium alkalo ds and other histamine liberators. *J Physiol* (Lond) 1951, **114**, 490—509

Finkelstein, M., S Y P'an, V N Niesler C. A. Johnson & J A Schneider On the pharmacology of oxyphencyclimine hydrochloride *J Pharmacol exp Ther* 1959, **125**, 330—338

Fitzgerald, J D Perspectives in adrenergic beta receptor blockade *Clin Pharmacol. Ther* 1969 **10**, 292—306

Foldes, F F., Erlof E G & Baart, N., J Zwartz & E. K. Zsigmond Inhibition of human cholinesterases by narcotic analgesics and their antagonists. *Arch int Pharmacodyn* 1959 **120**, 286—291

Fritz, M. E. & F P Brooks Control of bile flow in the cholecystectomized dog. *Amer J Physiol* 1963 **204**, 825—828

Gaddum J H & Z P Picarelli Two kinds of tryptamine receptor *Brit J Pharmacol* 1957 **12** 323—328

Gaensler E. A & J M. McGowan Biliary and small intestinal spasm due to synthetic analgesics methadone and methyldihydromorphinone (metopon) *Gastroenterology*, 1950 **16**, 484—489

Gaensler E. A., J M. McGowan & F F Henderson A comparative study of the act on of demerol and op um alkaloids in relation to biliary spasm. *Surgery* 1948, **23** 211—220

Gowdey C. W The change in pharmacological action produced by the introduction of a methyl group into priscol *Brit J Pharmacol* 1948, **3**, 254—262.

Green, A F Comparative effects of analgesics on pain threshold, respiratory frequency and gastrointestinal propulsion. *Brit J Pharmacol.* 1959 **14**, 26—34.

Bulbring, E Changes in configuration of spontaneously discharged spike potentials from smooth muscle of the guinea pig s *taenia coli* The effect of electronic currents and of adrenaline, acetylcholine and histamine *J Physiol* (Lond) 1957, **135**, 412—425

Burget, G E The regulation of the flow of bile *Amer J Physiol* 1925 **74**, 583—589

Burks T F & J P Long Catecholamine induced release of 5 hydroxytryptamine (5 HT) from perfused vasculature of isolated dog intestine *J pharm Sci* 1966 **55**, 1383—1386

Burks, T F & J P Long Release of intestinal 5 hydroxytryptamine by morphine and related agents *J Pharmacol exp Ther* 1967, **156**, 267—276

Burnstock, G & M E Holman Effect of drugs on smooth muscle *Ann Rev Pharmacol* 1966, **6**, 129—156

Butsch, W I, M McGowan & W Walters Clinical studies on the influence of certain drugs in relation to biliary pain and to the variations in intrabiliary pressure *Surg Gynec Obstet* 1936, **63**, 451—456

Cairnie, A B, H W Kosterlitz & D W Taylor Effect of morphine on some sympathetically innervated effectors *Brit J Pharmacol* 1961 **17**, 539—551

Carlyle, R F The mode of action of neostigmine and physostigmine on the guinea pig trachealis muscle *Brit J Pharmacol* 1963, **21**, 137—149

Caroli, J, P Porcher, Pequignot & M Delattre Contribution of cineradiography to study of the function of the human biliary tract *Amer J dig Dis* 1960, **5**, 677—696

Cox, B M & M Weinstock The effect of analgesic drugs on the release of acetyl choline from electrically stimulated guinea pig ileum *Brit J Pharmacol* 1966 **27**, 81—92

Crema, A & G Benzi Comportamento in vitro dello sfintere di Oddi di alcuni animali *Arch Fisiol* 1961, **60**, 374—386

Crema, A, G Benzi & F Berte The action of some natural substances on the terminal portion of the common bile duct isolated »in toto« *Arch int Pharmacodyn* 1962, **137**, 307—317

Crema, A & F Berte Action of sympathomimetic drugs on the isolated junction of the bile duct and duodenum *Brit J Pharmacol* 1963 **20**, 221—229

Curreri, A R & J W Gale Effect of analgesics and antispasmodics on common duct pressures *Ann Surg* 1950 **132**, 348—361

Dahl Iversen, E, A H Sorensen & E Westengaard Pressure measurement in the biliary tract in patients after cholecystolithotomy and in patients with dyskinesia *Acta chir scand* 1958 **114**, 181—190

Danhof, I E Pentazocine effects on gastrointestinal motor function in man *Amer J Gastroent* 1967 **48**, 295—310

Daniel, E E Effect of drugs on contractions of vertebrate smooth muscle *Ann Rev Pharmacol* 1964 **4**, 189—222

Daniel, E E & A Bogoch The mechanism of the spasmogenic action of morphine on the small intestine of man and dog Proceedings of the World Congress of Gastroenterology, The Williams & Wilkins Co, Baltimore 1958 138—155

Daniels B T F B McGlone & H E Shuey Extrahepatic bile duct motility *Amer J Gastroent* 1967, **48**, 198—203

Day, M, J R Vane An analysis of the direct and indirect actions of drugs on the isolated guinea pig ileum *Brit J Pharmacol* 1963 **20**, 150—170

Dollery, C T, J Peterson & M E Conolly Clinical pharmacology of beta receptor blocking drugs *Clin Pharmacol Ther* 1969, **10**, 765—799

Kozoll, D D & H Necheles A study of the mechanics of bile flow *Surg Gynec Obstet* 1942, 74, 961—967

Krantz, J C & C J Carr Pharmacological principles of medical practice 5th ed, The Williams & Wilkins Company, Baltimore 1961.

Kuschinsky, G & H Lullmann Über die Beziehungen der Barium Ionen zum Acetylcholin *Klin Wschr* 1950, 28, 137—138

Lab de Rech Mayvernay France Pat 1306610 (1962)

Lecchini, S, M Del Facca, G Soldani, G M Frigo & A Crema The actions of atropine, tropenziline and N butyl hyoscine bromide on the isolated distal colon of the guinea pig a comparison of their activities and mechanisms of action *J Pharm Pharmacol* 1969, 21, 662—667

Levis, G P The inhibition by morphine of the action of smooth muscle stimulants on the guinea pig intestine *Brit J Pharmacol* 1960, 15, 425—431

Liedberg G & C G A Persson Adrenoceptors in the cat choledochoduodenal junction studied in situ *Brit J Pharmacol* 1970, 39, 619—626

Long H Observations of choledochoduodenal mechanism and their bearing on physiology and pathology of biliary tract *Brit J Surg* 1942, 29, 422—437

Lueth, H C Studies on the flow of bile into the duodenum and the existence of a sphincter of Oddi *Amer J Physiol* 1931, 99, 237—252

Lullmann, H Über die synergistische Wirkung von Barium Ionen und Acetylcholin *Naunyn Schmiedebergs Arch exp Path Pharmak* 1950, 210, 111—118

Magee D F Physiology of gallbladder emptying In »The Biliary System«, Taylor, W, ed, Blackwell, Oxford, 1965, 233—247

Margolin, S Centrally mediated inhibition of gastrointestinal propulsive motility by morphine over a nonneural pathway *Proc Soc exp Biol (N Y)* 1963, 112, 311—315

Mattila, M J & J E Idanpaan Heikkila Modification by anticholinesterases of the uptake and release of ^{14}C-choline in electrically stimulated guinea pig ileum *Ann Med exp Fenn* 1968, 46, 85—88

Maxwell, R A Adrenergic blocking and adrenergic neuron blocking drugs In »Drill s Pharmacology in Medicine« (J R DiPalma, ed), 3rd edition, McGraw Hill Book Company 1965, 502—523

McGowan J M, W L Butsch & W Walters Pressure in the common bile duct of man *J Amer med Ass* 1936, 106, 2227—2230

McGowan, J M, P A Knepper, W Walters & A M Snell The relation of spasm of the second portion of the duodenum to biliary colic *Surg Gynec Obstet* 1938, 66, 979—986

Meltzer, S J The disturbance of the law of contrary innervation as a pathogenetic factor in the diseases of the bile ducts and the gall-bladder *Amer J med Sci* 1917, 153, 469—477

Menguy, R B, A Hallenbeck, J L Bollman & J H Grindlay Intraductal pressures and sphincter resistance in canine pancreatic and biliary ducts after various stimuli *Surg Gynec Obstet* 1958, 106, 306—320

Morris, T G, G F Sardi & S E Bradley Character of glucagon induced choleresis *Fed Proc* 1967, 26, 174

Murphree, H B Narcotic analgesics In »Drill's Pharmacology in Medicine« (J R Di Palma ed), 3rd ed, McGraw Hill Book Company, 1965, 246—273

Muscholl, E & M Vogt Secretory responses of extramedullary chromaffin tissue *Brit J Pharmacol* 1964, 22, 193—203

Grossman, M J Gastrin, cholecystokinin, and secretin act on one receptor *Lancet* 1970, **1**, 1088—1089

Gunter, M J, K S Kim, D F Magee, H Ralston & A C Ivy Choleretic potencies of some synthetic compounds *J Pharmacol exp Ther* 1950, **99**, 465—478

Hansson, K Experimental and clinical studies in aetiologic role of bile reflux in acute pancreatitis *Acta chir scand* 1967, suppl 375

Hansson, E, D T Masuoka & W G Clark Effect of nicotine on catecholamine and serotonin levels in vivo *Arch int Pharmacodyn* 1964, **149**, 153—160

Hicken, N F, A J McAllister, B J Franz & E Crowder Does the removal of the gallbladder produce function changes of the sphincter of Oddi and the choledochus? *Amer J Surg* 1951, **17**, 598—608

Hilton, J G The pressor response to neostigmine after ganglionic blockade *J Pharmacol exp Ther* 1961, **132**, 23—28

Hinshaw, J R, K E Hobler, A R Borja & C O Sahler Pentazocine, a potent non addicting analgesic *Amer J Med Sci* 1966, **251**, 57—62

Idanpaan Heikkila, J E Effect of some antitussives on the uptake and release of labelled choline and acetylcholine in electrically stimulated guinea pig ileum *Ann Med exp Fenn* 1968, **46**, 193—200

Ivy, A C The physiology of the gallbladder *Physiol Rev* 1934, **14**, 1—102

Ivy, A C & L Goldman Physiology of biliary tract *J Amer med Ass* 1939, **113**, 2413—2417

Jacobsson, B Determination of pressure in the common bile duct at and after operation *Acta chir scand* 1957, **113**, 483—488

Jacobsson, B, L O Lanner & C Rådberg Dynamiken i distala delen av choledochus studerad genom filmning under tryckregistrering *Nord Med* 1957, **58**, 1553

Jacobsson, B, L O Lanner & C Rådberg The dynamic variability of the cholelocho pancreatico duodenal junction *Acta radiol* (Stockh) 1959, **52**, 269—281

Jaffe, J H Narcotic analgesics In »The pharmacological basis of therapeutics» (Goodman, L S & A Gilman, ed), 3rd edition, Macmillan Company, New York, 1965, 247—284

Johnson, F E Effect upon the biliary tract of sectioning the splanchnic nerves *Proc Soc exp Biol (N Y)* 1941, **47**, 399—400

Johnson, E E & E A Boyden The effect of double vagotomy on the motor activity of the human gall bladder *Surgery* 1952, **32**, 591—603

Jorpes, E, V Mutt, G Jonson, L Thulin & L Sundman The influence of secretin and cholecystokinin on bile flow In »The Biliary System», Taylor, W, ed Blackwell Oxford 1965 293—301

Kharkevich, D A Ganglion blocking and ganglion stimulating agents W D M Daton, ed, Pergamon Press 1967

Kipp, H A Observations on the variations in bile pressure in the human biliary tract *J Amer med Ass* 1936, **106**, 2223—2230

Kjellgren, K The influence of morphine and pethidine in combination with leval lorphan on biliary duct pressure after cholecystectomy *Brit J Anaesth* 1960 **32**, 2—6

Kosterlitz H W & G M Lees Pharmacological analysis of intrinsic intestinal reflexes *Pharmacol Rev* 1964, **16**, 301—339

Kosterlitz, H W & J A Robinson The inhibitory action of morphine on the contraction of the longitudinal muscle coat of the isolated guinea pig ileum *Brit J Pharmacol* 1958 **13**, 296—303

Szerb, J C The effect of morphine on the adrenergic nerves of the isolated guinea pig jejunum *Brit J Pharmacol* 1961, 16, 23—31

Taeschler, M, H Konzett & A Cerletti Zur Pharmakologie von Palerol *Schweiz med Wschr* 1960 90, 136—140

Takagi, K, & I Takayanagi Barium receptor on intestinal smooth muscle Mode of action of morphine on the papaverine like antispasmodic action *Arch in Pharmacodyn* 1962, 135, 223—234

Tammisto, T, A Jaattela & S Takki Effect of pentazocine and pethidine on plasma catecholamine levels *Scand J clin Lab Invest* 1970, 25, suppl 113, 94

Trendelenburg U The action of morphine on the superior cervical ganglion and on the nictitating membrane of the cat *Brit J Pharmacol* 1957, 12, 79—85

Toh, C C Experiments on the barium contraction of the guinea pig's ileum *J Physiol* (Lond) 1951, 114, 33P

Vagne, W & M J Grossman Cholecystokinetic potency of gastrointestinal hormones and related peptides *Amer J physiol* 1968, 215, 881—884

Walker W F Vertebrate Dissection, 3rd ed, W B Saunders Company, Philadelphia 1965

Westphal K *Muskelfunktion, Nervensystem u Pathologie der Gallenwege* *Z klin Med* 1923, 96, 22—150

Wick H Pharmakologie des Buscopan *Naunyn Schmiedebergs Arch exp Path Pharmak* 1951, 213, 485—500

Williams, R D, J C Fish & D D Williams The significance of biliary pressure *Arch Surg* 1967, 96, 374—379

Young D C, R A Vander Ploeg R M Featherstone & L G Gross The interrelationships among the central peripheral and anticholinesterase effects of some morphinan derivatives *J Pharmacol exp Ther* 1955, 114, 33—37

Zaterka, S & M I Grossman The effect of gastrin and histamine on secretion of bile *Gastroenterology* 1966, 50, 500—505

Zeppa, R, D C Grossekreutz & K Sugioka Histamine release into the circulation by meperidine (Demerol®) *Proc Soc exp Biol (N Y)* 1961, 106, 794—796

Ablad, B M Brogård & L Ek Pharmacological properties of H 56/28 a β adrenergic receptor antagonist *Acta pharmacol* (Kbh) 1967, suppl 2, 9—40

Myers, R N, G J Haupt, N C Birkhead & J M Beaver Cinefluorographic observations of common bile duct physiology *Ann Surg* 1962 **156**, 442—450

Necheles, H, W Scruggs, S Kraft & W H Olson In vivo inhibition of barium stimulated intestinal motility by atropine *J Pharmacol exp Ther* 1953, **107**, 61—68

Oddi, R D une disposition a' sphincter spéciale de l ouverture du canal choledoque *Arch ital Biol* 1887, **8**, 317—322

Orth, O S General anesthesia I Volatile agents In »Drill s Pharmacology in Medicine« (J R DiPalma, ed), 3rd ed, McGraw Hill Book Company, 1965 100—115

Pallin, B & S Skoglund On the nervous regulation of the biliary system in the cat *Acta physiol scand* 1961, **51**, 187—192

Parry, E W, G A Hallenbeck & J H Grindlay Pressures in the pancreatic and common ducts *Arch Surg* 1955, **70**, 757—765

Paton, W D M The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea pig ileum *Brit J Pharmacol* 1957, **12**, 119—127

Potter, J C & F C Mann Pressure changes in the biliary tract *Amer J med Sci* 1926, **171**, 202—217

Reach F Der Schliessmuskel des Ductus choledochus in funktioneller Beziehung *Naunyn Schmiedebergs Arch exp Path Pharmak* 1920, **85**, 178—198

Reich H A new procedure for the relief of biliary dyskinesia *Surg Gynec Obstet* 1940, **71**, 39—43

Reynell, P C & G H Spray The effect of ether and pentobarbitone sodium on gastrointestinal function in the intact rat *Brit J Pharmacol* 1957, **12**, 104—106

Ritter, U Studien der Funktion und des Schliessungsdruckes des Sphincter Oddi unter verschiedenen pathophysiologischen Dedingungen *Klin Wschr* 1956 **34**, 756—759

Robelet A, N Bizard Gregoire & J Bizard The action of dextromoramide on the sphincter of Oddi In »The Biliary System«, Taylor, W ed Blackwell, Oxford 1965, 335—339

Sandblom, Ph W L Voegtlin & A C Ivy The effect of cholecystokinin on the choledochoduodenal mechanism (sphincter of Oddi) *Amer J Physiol* 1935 **113**, 175—180

Schachter, M The release of histamine by pethidine atropine quinine and other drugs *Brit J Pharmacol* 1952 **7**, 646—654

Schaumann, W Zur Wirkung der morphinahnlich wirkenden Analgetica auf den Meerschweinchendunndarm in situ *Naunyn Schmiedebergs Arch exp Path Pharmak* 1954, **223**, 348—354

Schaumann W Inhibition by morphine of the release of acetylcholine from the intestine of the guinea pig *Brit J Pharmacol* 1957 **12**, 115—118

Shi, K The influence of the gall bladder, Oddi s muscle and the duodenum upon th outflow of bile Report I Injection of visceral nerve poisons and pituitrin *Jap J Gastroent* 1933 **5**, 19—25

Snell, A M, J M McGowan & W L Butsch Colics following cholecystectomy The probable mechanism of their production *R I med J* 1936 **19**, 113—119

Stalport, J, L Nicolas, A Demelenne & G Horeczki Sur la pharmacologie de la voie biliaire principale *Arch int Pharmacodyn* 1959 **119**, 142—161

Stening, G F & M J Grossman Potentiation of cholecystokinetic action of cholecystokinin (CCK) by secretin *Clin Res* 1969 **17**, 528

Stille, G & H Hilfiker Zur Wirkung von Pharmaka auf das Gallengangssystem *Arzneimittel Forsch* 1962, **12**, 461—465

Szerb, J C The effect of morphine on the adrenergic nerves of the isolated guinea pig jejunum *Brit J Pharmacol* 1961, 16, 23—31

Taeschler, M, H Konzett & A Cerletti Zur Pharmakologie von Palerol *Schweiz med Wschr* 1960, 90, 136—140

Takagi, K., & I Takayanagi Barium receptor on intestinal smooth muscle Mode of action of morphine on the papaverine like antispasmodic action *Arch in Pharmacodyn* 1962, 135, 223—234

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Trendelenburg U The action of morphine on the superior cervical ganglion and on the nictitating membrane of the cat *Brit J Pharmacol* 1957, 12, 79—85

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Williams, R. D., J C Fish & D D Williams The significance of biliary pressure *Arch Surg* 1967, 95, 374—379

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Zaterka, S & M I Grossman The effect of gastrin and histamine on secretion of bile *Gastroenterology* 1966 50, 500—505

Zeppa, R. D C Grossekreutz & K Sugioka Histamine release into the circulation by meperidine (Demerol®) *Proc Soc exp Biol (N Y)* 1961, 106, 794—796

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PREFACE

In late 1969 the Section of Drug Research of the Swedish Medical Society and RUFI, the Association of Representatives of Foreign Pharmaceutical Industries in Sweden decided to sponsor a symposium entitled "Biological and pharmaceutical aspects of pharmacokinetics and therapeutics". The symposium took place on June 5–6, 1970, in Stockholm. The purpose was a dual one, to fulfill scientific as well as educational ambitions. The proceedings were accompanied by useful interdisciplinary discussions of the pharmaceutical, pharmacological and clinical aspects of drug therapy.

Many of the speakers selected for this symposium are eminent scientists and the organizing committee acknowledges that practically all active participants have contributed to this volume with their manuscripts. Some have even extended their formal presentations into rather extensive reviews of their research fields.

Emphasis has been put on the clinical importance of interindividual differences (in e.g. renal elimination and drug metabolism) which determine the biological availability of drugs given to patients.

It is a prediction that a much more effective, yet safe, drug therapy can be initiated, not only in the future but today, by taking advantage of the many facts brought together in this volume. A prerequisite will be that health authorities, the academic community and the drug industry collaborate and jointly support those specialists in pharmacy, pharmacology and therapeutics that try to work for more rational therapeutics in the hospital environment.

Percy Lindgren, Roland Olsson, Sten Sandberg, Folke Sjoqvist

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PHYSICO CHEMICAL FACTORS INFLUENCING *PHARMACOKINETICS*

PHYSIOLOGICAL AND BIOCHEMICAL FACTORS INFLUENCING PHARMACOKINETICS

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The Physicochemical and Pharmacokinetic Bases for the Biopharmaceutical Evaluation of Drug Biological Availability in Pharmaceutical Formulations

By

Edward R. Garrett

Abstract The *operational determination* of the bioavailability of a vital drug from its pharmaceutical formulation by the extent and rate of its appearance in the blood and tissues of an intact biological organism is termed *biopharmaceutics*. Such bioavailability is a complex function of the physicochemical factors that determine its stability in the formulation and in the biological fluids, the rate *of release of drug particles from, or disintegration of, the formulation matrix*, the rate of dissolution of the dispersed drug particles as affected by the pH, volume and agitation of the dissolution media, the solubility of the drug in the solvent, its transport to the absorption site, the pKa of the dissolved substance that determines the concentration of neutral species at the pH at the site, the partition of dissolved drug into the lipid-like membranes and the subsequent rate of diffusion into the body compartments. *Pharmacokinetics* determines the amounts of drug absorbed, the rate of absorption and release from the pharmaceutical formulation in the intact biological system. Experimental data may be obtained from amounts of drug and/or metabolites monitored in the urine, faeces and blood as functions of time. Total amounts excreted and areas under blood level time curves with their defined limitations are one set of criteria of bioavailability. Rates of absorption, deduced from *rates of change of blood level and excretion*, form another set of criteria whose various limitations are also defined. Dissolution rate studies have limited *a priori* value in predicting bioavailability but may be useful in quality control procedures when correlated with biopharmaceutical data. Although many mathematical functions may be operationally useful in characterizing *in vitro* dissolution rates of drugs from dosage forms, the most valid presentation of data is the percentage og drug released at various time intervals.

A drug is a chemical substance that acts in a biological system to preferentially give a beneficial pharmacological or chemotherapeutic effect. However, it is almost impossible to administer the requisite numbers of pure molecules of drug at the site of this action in the amount necessary to maintain the desired concentration for the necessary time interval to accomplish this function. The molecules must reach the sites of action in the complex organism to be effective and almost always must be in-

CLINICAL IMPLICATIONS OF PHARMACOKINETICS

the metabolic and pharmacokinetic transformations of the drug Oral routes are preferred in many societies, but the nature of the drug action must be considered for an appropriate choice For example, an anti emetic must be administered by an alternate route than oral

A complex organism such as man has few direct routes of entry for a drug without puncturing (i e parenterally administering, intramuscularly or intravenously) or diffusing (topically administering) through the skin covering he is endowed with These center about the pharyngo-laryngeal gastro-intestinalrectal tract and a drug administered orally has the nasty habit of running this gamut, the extent of which is a function of its chemistry and physics The actual absorptions from this tract do not all follow the same pathways Pulmonary, buccal and rectal absorption in richly endowed capillary beds permit the drug to bypass the liver whereas gastro-intestinal absorption gives the liver an opportunity to metabolize a drug in high concentrations before it is poured into the mainstreams of the body A drug whose action depends on the instantaneous blood level and is diluted by the compartments of distribution (thiopental, thiomebumalum NFN) needs an instantaneous mode of absorption into the blood A drug that has a high renal clearance or an active tubular secretion (penicillin) needs a delayed absorption

Absorption of drugs from solution

However, *a priori* design of a dosage form must consider those factors which permit availability to the absorption site Maximum availability would be manifested when the administered dose is in solution and in high con*centration* The limitations would be natural ones the solubility of the drug its ionic character, the maintenance of its stability, *the ability of the tissue to which it is applied to absorb it into the body* The optimal presentation of the drug molecule is as an uncharged species in solution and thus the pKa of the drug, its concentrations, solubility and pH at the absorption site determine the effective concentration gradient (BRODIE 1964 BRODIE & HOGBEN 1957, HOGBEN *et al* 1957 & 1959, SCHANKER 1959, 1960, 1961 & 1962, SCHANKER *et al* 1957 & 1958, SHORE *et al* 1957) *Water insoluble materials*, such as antabuse (disulfiramum NFN) *need high dosages* to obtain the small amounts absorbed by oral administration

If passive absorption processes are postulated, the *rates of diffusion* through the biological lipoid like barrier are *dependent on the* lipid/water partition coefficient of the *uncharged drug*, *i e*, *the ratio* of the thermodynamic activities in both phases *Drugs that* do not partition into an oil phase such as dialkylated amides, *may have* activity on parenteral administration but not on oral *administration* (GARRETT & WEBER 1962)

troduced in a physical form or vehicle which may modify the transport to the biophase compartment wherein the drug molecules react with these receptor sites These forms and vehicles with their necessary excipients and constitute the pharmaceutical formulations that are actually administered A simple solution at an exact or ambient pH value is just as much a possibly elegant formulation as the powdered crystals of the drug, or a complex admixture of pharmacologically inert and active ingredients Each formulation modifies the release and availability of the drug molecules to absorption sites, the first steps in their long traverse to the receptor sites of drug action

The effectiveness of drug action in the complex organism is not only limited by the affinity or reactivity of the drug molecules with the receptor sites and the release of the drug from the formulation There are other major factors that determine drug action and which depend on the number of the administered drug molecules that reach the site of action and how long they remain there The necessary vital processes of the body delay the transport of drug molecules across membranes, dilute them into various compartments of distribution, transform them into metabolites and excrete them Thus, if a time duration of drug presence in a biophase compartment is vital for the cure of disease, the mode, frequency and dose of administration must be appropriately modified and schedule The chemical modifications of an active principle will also modify these transport and metabolic processes, since a drug is a chemical that not only acts *in* and *on* a biological system, but is acted on *by* the biological system Since a time duration of drug presence at a site of action is vital for the cure of disease, a comprehensive and quantitative expression of these time courses of drug distribution as a function of dose and routes of administration is necessary for the establishment of proper dosage regimens for the cure of disease and the avoidance of toxicities

Pharmacokinetics is the study of the time courses of absorption, distribution, metabolism and excretion of drug in the intact total organism The amounts of drug and metabolites in available compartments in the body such as blood, tissues, and excreta are measured as functions of time and dosage Kinetic models are established to quantitatively predict these dynamic processes The derived kinetic constants and quantifiable concepts, such as apparent volumes of distribution, are related to physiological and clinical reality

Drug properties and routes of administration

The choice of the route of administration, the dose and the intervals of dosing are dictated by the nature of the disease and the drug the cultural patterns of the society, the restrictions of the technology and

If the drug to be administered is active by itself and not as the metabolite, if metabolism by the liver is not instantaneous or excessive, and if it is gastro-intestinally absorbed, oral administration is indicated in accordance with established theories on the lipid like nature of the gastro intestinal tract where non ionized neutral molecules are preferentially absorbed (BRODIE 1964, BRODIE & HOGBEN 1957, HOGBEN *et al* 1957 & 1959, SCHANKER 1959, 1960, 1961 & 1962, SCHANKER *et al* 1957 & 1958, SHORE *et al* 1957) Strong acids of low pKa are preferentially absorbed from the stomach whereas protonated amines are difficultly absorbed at this site Conversely, the non protonated amines are more readily absorbed from the more alkaline intestine whereas the anions of organic acids are not The pKa of the amine (or acid) dictates the effective concentration, αC (or $[1 - \alpha]C$) of the neutral absorbed species at the pH of the absorption site where C is the total concentration and α is the degree of dissociation in

$$pH = pKa - \log\frac{\alpha}{1 - \alpha} \qquad \text{(Eq 2)}$$

However, it is not only the ability of the drug to be transported to the site of absorption in sufficient amount (a factor dependent on the interaction of the solubility and pKa of the drug with the pH of the fluids bathing that site) that determines absorption It is not only the ability of the drug to diffuse through the membrane, a factor dependent on the parameters of Fick s law given in Eq 1 A most important item is the ability of the drug to be transferred into the gastro-intestinal wall which is dependent on the apparent partition coefficient between the aqueous phase and the lipid like gastro-intestinal mucosa The greater the oil/water distribution coefficient, the greater the rate of diffusion if molecular size and steric factors are equivalent The rate of passive gastro-intestinal absorption for negligible concentrations of drug in the desorbing solution (i e $C_2 \sim O$) may be characterized by

$$Rate = DP(1 - \alpha)C_1 \qquad \text{(Eq 3)}$$

where D is the apparent specific absorption rate for a given length of gastro-intestinal tract, α is the degree of dissociation for the pH at the site of absorption P is the oil/water partition coefficient for intestinal mucosa and $C_1 = C'_1/P$ is the actual concentration of the drug in all its ionic forms This D value is related to D' of Eq 1 by $D = D'A/x$ In an homologous series of drugs, rates of absorption through lipid-like membranes will tend to increase with increases in the oil/water partition coefficient.

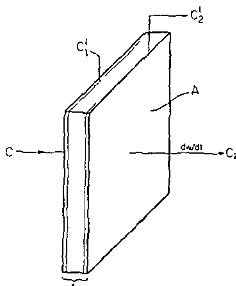


Fig 1 Schematic for the steady state rate of diffusion, dw/dt, through an X thickness of membrane of area A where C_1' is the concentration in the membrane monolayer adjacent to the diffusing solution of total concentration C_1, and where C_2 is the concentration in the membrane monolayer adjacent to the desorbing solution of concentration, C_2 An instantaneous equilibrium characterized by the apparent partition constant, P, is established between the neutral species of C_2 and C_2' and between the neutral species of C_1 and C_1' so that if α_1 and α_2 are the degrees of dissociation of the drug in the absorbing and desorbing solutions respectively

$$dw/dt = \frac{D'A}{X}\left[C_1' - C_2\right] = \frac{D\,AP}{X}\left[(1-\alpha_1)C_1 - (1-\alpha_2)C_2\right]$$

where D is the diffusivity constant

The classical diffusional factors of area increasing, thickness of membrane decreasing and molecular weight and steric factors modifying absorption rate must be considered

The rates of diffusion through a membrane can be characterized by a version of Fick's law (see fig 1)

$$\text{Rate of diffusion} = \frac{D'A}{x}(C'_1 - C'_2) \qquad \text{(Eq 1)}$$

where A is the surface area, x is the thickness of the membrane, C'_1 is the effective concentration of the drug in its diffusable form in the membrane monolayer adjacent to the diffusing solution, C'_2 is the effective concentration of the drug in its diffusible form in the membrane monolayer adjacent to the desorbing solution, and D' is the diffusion constant for the substance in the membrane This latter is undoubtedly a function of the drug's molecular weight, stereochemistry and energies of solvation with the molecular constituents of the membrane

A secondary proof of availability is the appearance of the drug and its metabolites in the blood, tissues and excreta of the organism This is based on the reasonable assumptions that the drug will exercise its action in the body when it does appear therein and that the biological activity is related to the amounts that do

The evaluation of this "biological availability" of the drug from its pharmaceutical formulation in an intact biological system is termed *biopharmaceutics* This term encompasses those applications of pharmacokinetics to determine the amounts of drug absorbed, the rates of absorption, and the rates of release from the pharmaceutical formulation *in the intact biological system* This is frequently accomplished by comparing the time course of drug concentrations in the body with the most readily available formulations of the drug which are, if possible, intravenously and orally administered solutions Comparisons of the biological availability of the drug from various dosage forms can be made on the basis of the relative amounts in the different biological compartments as a function of time

Amounts of drugs and metabolites in excreta as criteria of amounts absorbed

The simplest way to determine the amount of a drug biologically available from a formulation is to measure the amount of drug or its metabolites that are excreted from the body by routes other than those that are simple consequences of the route of administration Even these simple procedures have limitations Obviously, drug in the faeces is not a valid criterion of an absorptive process if the drug were orally administered The issue would be highly confused if biliary excretion were the major route of elimination

The use of amounts excreted as a criterion of relative biological availability depends on the acceptance of the assumption that the amounts of drug and its metabolites that are excreted in the urine are directly proportional to the amount absorbed Comparisons of biological availability from different routes of administration by excretory methods can only be valid if the amounts of unchanged drug and the metabolites excreted are obtained The use of the amount of only one metabolite excreted as a criterion of efficacy of absorption, even on the assumption that the percent metabolized by a particular route is constant and independent of dose, is invalid if the drug is metabolized or transformed before absorption A case in point would be where gastro-intestinal degradation or enzymic modification of a drug before absorption would give a higher fraction of the total dose excreted as the metabolite than when the drug was intravenously administered. Since fractions of the dose metabolized may differ

Degradation in vivo and biological availability

The potential degradation of a drug in the fluids of the gastro intestinal tract and other fluids of the body must be considered The muscle relaxant, p-chlorobenzaldehyde oxime, parenterally active but orally inactive, is readily solvolyzed to inactive p-chlorobenzaldehyde by the acid content of the stomach (GARRETT 1962), the antibiotic streptozotocin (GARRETT 1960) and the antileukaemic N-nitrosoureas (GARRETT *et al* 1965) are highly unstable at the pH values of the intestine and blood The former problem may be solved by enteric coating, the latter by administration parenterally, or orally on a full stomach

Degradation in vitro and biological availability

The degradation of a drug in the formulation is certainly an important factor in determining the net biological availability Most significant degradation processes are solvolytic (GARERTT 1967) and are functions of pH and drug concentration in solution, e g solubility However, some are peculiar results of inappropriate choices of pharmaceutical adjuvants and excipiets For example, the presence of an alkaline lubricant, sodium stearate, liberated free amine from its hydrochloride This subsequently reacted with the excipient lactose in the ' browning reaction" in the absorbed moisture to result in discolored preparations (CASTELLO & MATTOCKS 1962) Magnesium stearate changed the microscopic pH environment of acetylsalicylic acid and promoted its solvolytic breakdown (RIBEIRO *et al* 1955)

The major route of less of phenylephrine (metaoxedrinum NFN) activity in tablet formulations containing aspirin was due to the breakdown of aspirin to produce an acylating function at room temperature The primary degradation pathway was the acetylations of the secondary amine function At elevated temperatures, acetylation of the phenolic and alcoholic group of phenylnephrine also occurs (TROUP & MITCHINER 1964)

It has been shown that bisulphite added as a preservative to epinephrine (adrenalinum NFN) formed an addition compound wherein the bio logical activity was inactivated (SCHROETER *et al* 1958) Procaine was inactivated in solution on reaction with glucose to form a procaine N-glycoside (CANNELL 1951)

Criteria for biological availability

The primary proof of pharmacological or chemotherapeutic availability of a drug at site of action from its vehicle or formulation is the magnitude and duration of the biological response Unfortunately, these are difficult to quantify The mathematical delineation of a valid pharmacological or chemotherapeutic response in a complex organism is a *difficult process*

concentration of the drug in the blood against time, A, is directly proportional to the absorbed fraction, γ, of the dose, D_o, and inversely proportional to the overall apparent first order elimination constant, k_e, the sum of the first order rate constants of the separate eliminations and metabolisms, and to the apparent equilibrated volume of distribution, V_f of the body

$$A = \gamma D_o / V_f k_e \qquad \text{(Eq 4)}$$

This equation is valid to compare availability of a drug by any route of administration and any order of absorption This also includes the zero order case where the drug is infused at a constant rate The only prerequisite is that the drug is not preferentially metabolized before absorption by one administrative route over another

If the drug is highly metabolized, oral absorption differs from administration by most other routes in that an extremely high concentration of drug hits the liver on the first pass prior to its dilution with the blood and equilibrating tissues The net result would be that even though all the drug is absorbed, a greater fraction is metabolized initially on oral absorption and the areas under blood level-time curves for drug *cannot* be equal for oral and intravenous administration

Thus, it is best to compare the relative biological availability of drug in various formulations administered by the same route A primary standard would be a solution of the drug, and oral availability from a tablet could be compared to the availability from a solution If the insolubility of the material contraindicates the use of a solution standard, then an equivalent amount of drug in the most readily available formulation would be the proper choice Perhaps an aqueous suspension or a quickly disintegrating tablet of amorphous or micronized material containing a proper wetting agent would be an appropriate choice The preparation of such formulations that could appropriately surrogate as standards which retain their stability and release rates on storage will obviously be a problem in the establishment of biopharmaceutical procedures

The application of Eq 4 to the Dost Method of corresponding areas (DOST 1968) is also limited in that all eliminations and metabolisms must be first order and the pertinent rate constants must be independent of dose (GARRETT 1971) If this is not so, if enzymes or proteins are saturable, if the capacity of a pharmacokinetic compartment is limited, if the renal processes of filtration, secretion, or absorption vary as a function of *free* drug concentration in the blood, then relations among areas of such curves for different absorption efficiencies for the same dose in the *same animal* are more complicated and the direct application of Eq 4 is invalid (DOST 1968)

among individuals, it is always best to use an individual as his own control when comparing total drug or metabolite excreted for estimation of relative biological availabilities of several formulations administered by the same route

Amounts of drugs and metabolites in blood as criteria of amounts absorbed

An alternative method of determining the amount of drug absorbed from the formulation is by the application of the Dost principle of corresponding areas (Dost 1968). If a drug is eliminated from, or metabolized in the body by a first order process, the area under the plot (fig 2) of the

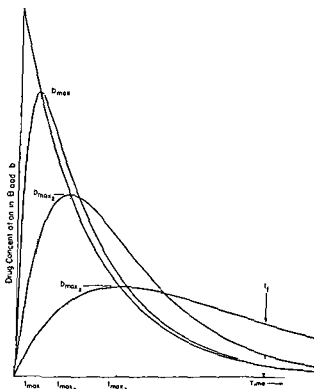


Fig 2 Plots of concentrations in blood for the sequential first order pharmacokinetic model Gastro intestinal tract $\xrightarrow{k_a}$ equilibrated compartments of distribution $\xrightarrow{k_e}$ excreta *and metabolites, where k_e is maintained constant and various k_a values are used* This is valid when different formulations of different release rates are administered orally to the same person where the invariance of the overall *elimination rate constant,* k_e, and the apparent equilibrated volume of distribution, V_f, can be assumed for the postulated one compartment body model The areas, A, under each curve are equal and are $A = \gamma D_0/k_e V_f$ where γD_0 is the fraction, γ, of the dose, D_0, administered that is absorbed This is true no matter the order or functional dependence of the absorption rate If areas under such curves are only obtained up to a finite time, e g, t_f, rather than up to infinite time, it is apparent that such areas from different formulations *will not be equal even when equivalent* amounts of drug are absorbed

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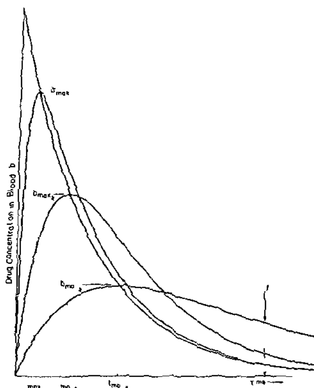


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mulation may permit a finite amount of drug (or metabolite) in the *blood* at the time interval chosen

If a "deep" compartment of reasonable capacity, such as *fat, liver* or bone, exists in the organism for a specific drug, *a sustained or* slowly *releasing* dosage form would transfer a greater *amount of the drug* to such a compartment than the equivalently dosed, quickly absorbed dosage form (GARRETT 1971) This would not only *mean* that blood levels have to be monitored for highly prolonged times in this case but also with sufficient *precision* so that satisfactory estimates of the amounts "dribbled" *out of the body* for these prolonged times would not permit underestimation of the amounts absorbed by the application of the Dost principle and their pertinent Eqs 4–6

A possible design to determine relative biological availabilities by these methods initially would require the determination of the reproducibility of such areas within an individual on repetitive dosing of several levels of a standard solution Reproducibility of estimates of biological availability on repetitive dosing of representative formulations would also be desirable The resultant statistics in several individuals should permit the determination of the reliability of this method where an individual is used as his own control The need is to determine the error of estimate of relative biological availability *in an individual and not among individuals* A probable proper and practical final design would be the use of four normal persons to study the biological availability of the standard, S, and the formulation, F, by excretory or blood level methods in the typical sequences S,S,F, F F,S, S F,S, F,S,F

Rates of absorption as determining factors in bioavailability

The *amount* of drug available from a formulation is not the only important factor in determining the bioavailability of the drug from the dosage form Certainly the availability of a reproducible amount of drug, which may only be a constant fraction of the dose in a formulation, within a *specified time interval is* just as proper a basis for an effective non toxic dosage *regimen as a formulation where the dose* is totally available The major reason total availability is desirable is that it is difficult for reasons of biological variability to obtain reproducible *fractional releases* of the dose among individuals

More important criteria for a dosage regimen in many instances are the *rates* of the drug being made available in the body It may be desirable to maintain a body level of drug for a desired length of time *below* the maximum *level* which yields toxicity and *above* the minimum level which *exercises no effect* against a disease This it not necessarily a sustained blood *level within* specified limits since the efficacy of some drugs may

The concentration of metabolite in the blood may be used to determine areas under blood level time curves and will also conform to Eq 4 provided that the k_e, which now represents the elimination of this metabolite, is first order and independent of dose The limitations must be that the fraction, α, of the fraction γ of drug absorbed that is converted to the metabolite is constant and the γ value of Eq 4 must be multiplied by α and supplanted by $\gamma' = \alpha\gamma$

Although Eq 4 is valid for the comparisons of biological availability of different formulations when the study of each is conducted in separate individuals, it needs the definitive evaluation (DOST 1968) of the overall apparent first order rate constants, $(k_e)_1$ and $(k_e)_2$, and the apparent equilibrated volumes of distribution, $(V_f)_1$ and $(V_f)_2$, for the respective individuals used so that the relative absorptions γ_1/γ_2 of same amount of drug D_o in formulation 1 and formulation 2 may be estimated from the ratio of the areas as

$$\gamma_1/\gamma_2 = (A_1/A_2)\,(k_e)_1\,(V_f)_1/(k_e)_2(V_f)_2 \qquad \text{(Eq 5)}$$

This also assumes that the fraction of each formulation absorbed is independent of the individual This is an unwise assumption since gastrointestinal absorption efficacy varies widely among individuals

Thus, it is the wisest procedure to determine the relative amounts of various formulations absorbed by using each person as his own control This accepts the more reasonable postulates that the V_f, k_e, absorption efficiency and also α (if a metabolite is monitored instead of the original drug) are constant and invariant within the same individual Thus, the relative biological availability of formulations 1 and 2 are, from Eq 5 simply the ratios of the areas under the blood level-time curves,

$$\gamma_1/\gamma_2 = A_1/A_2 \qquad \text{(Eq 6)}$$

The proper application of Eq 4 is when the areas are determined for blood level-time curves that are conducted *to those times when there* are negligible concentrations of drug (or metabolite if it is the measured species) in the blood If the areas under such curves are only obtained *up to a finite and arbitrary* time interval, the methods based on Eqs 4–6 are not applicable even when all the previously stated prerequisites of these equations are met (fig 2) Obviously, the absorption phase must be completed for the several formulations to be compared. Although the total areas under the curves for a sustained or delayed release medication that delivers the same amount of drug to the central body compartment as a quickly *released medication* may be equal, the delayed release for-

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be improved by successive spikes of concentrations in the body This is not necessarily a blood level at all, but may be a tissue level which is not exactly parallel to the blood level.

Nevertheless, the rate of appearance, concentration and duration of the requisite concentration of the drug in the biophase of drug action must be related to the rate of appearance of the drug in the blood and its rapidly equilibrated tissues Thus, the parameters that can characterize this latter can be related to the appearance of the drug in the biophase and the desired pharmacological action Since it is frequently technologically infeasible to monitor the loss of drug from its formulation at an absorption site, the rate of absorption of the drug as derived from the drug's appearance in the equilibrated fluids of distribution of the body is a proper criterion of bioavailability

'Feathering as a method of estimation of rate of absorption and drug release from a formulation

The simplest model to establish for the appearance of a drug in the body from a formulation is the one compartment body model with first order release of the amount F in the formulation, first order absorption of the amount D and overall first order transformation of the amount B in the body of apparent volume of distribution V_f into the urine U and metabolites M



$$F \xrightarrow{k_f} D \xrightarrow{k_a} B \xrightarrow{k_e} U + M \qquad \text{(Eq 7)}$$

where the respective apparent first order rate constants are k_f, k_a, k_e In the specific instance when a solution of the drug is administered $k_f = O$ If, as is generally, but not always true (penicillin is a classical example of the exception) the drug is absorbed *more readily than it is excreted, as* in the example of fig 3) a plot of the logarithm of the concentration, $b = B/V_f$, of drug in the blood against time would permit estimation (DOST 1968) of the overall elimination rate constant, k_e, from the terminal slope (fig 3) of such a plot

The intercept of the line drawn to the terminal data of this plot is log $\frac{\gamma_a D_o}{V_f}\frac{k_a}{(k_a - k_e)}$ where γ_a is the fraction of the dose D_o absorbed

The plot of the logarithm of the amount of the drug yet to be excreted *into the urine, log* $(U_\infty - U)$ against time should also be linear after the drug is absorbed and the overall elimination rate constant, k_e, should also be obtainable from this slope (fig 3)

The extrapolation of the terminal linear segment of the semilogarithmic plot of blood concentration, b (or amount of drug in the body at constant

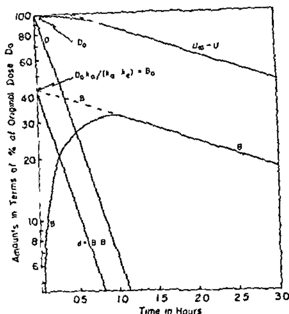


Fig 3 Semi logarithmic plots of amounts of drug in the blood, $B \approx bV_f$, and not-yet excreted into the urine, $U_\infty - U$, in accordance with the model $D \xrightarrow{k_a} B \xrightarrow{k_e} U$ The total dose, D_0, is considered to be absorbed in this example, i.e. $\gamma_A = 1$. The terminal slopes of these plots of ln B and ln ($U_\infty - U$) against time are $-k_e$ "Feathering" gives the difference between the linearly extrapolated values of the terminal data and the real data, i e., $d = B' - B$ which when plotted semi logarithmically i e, as ln d *versus* t produces the straight line with the slope $- k_a$ This would be the same slope that would have been obtained if the drug, D, released by a first order rate from the absorption site had been plotted as ln D *versus* t.

V_f i e B) against time is feasible and produces values of the curve, b' (or B' in fig 3) when the ratio of the first order rate constant for absorption to that of elimination, k_a k_e, is approximately ten or greater If the absorption rate constant is less than this magnitude, absorption continues during the period of decay of drug content in the blood and the rate of decrease in blood level is a summation of absorption and elimination effects Under these circumstances the plot of logarithms of drug concentrations in the blood (or of amounts of drug not yet excreted into the urine) does not become completely terminally linear and the apparent slope of the terminal line underestimates the true rate constant of elimination, k_e, as long as there is significant contributions to the body from the absorption process.

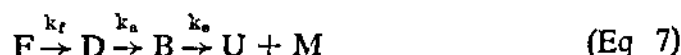
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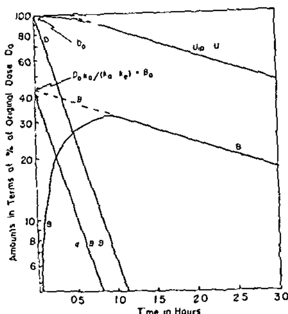


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V_f, i e B) against time is *feasible* and produces values of the curve, b' (or B' in fig 3) when the ratio of the first order rate constant for absorption to that of elimination, k_a k_e, is *approximately ten or greater* If the absorption rate constant is less than this magnitude, absorption continues during the period of decay of drug content in the blood and the rate of decrease in blood level is a summation of absorption and elimination effects Under these circumstances the plot of logarithms of drug concentrations in the blood (or of amounts of drug not yet excreted into the urine) does not become completely terminally linear and the apparent slope of the terminal line underestimates the true rate constant of elimination, k_e, as long as there is significant contributions to the body from the absorption process.

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extrapolation of this linear segment to times when the drug is being absorbed is feasible and values of B' or b' can be estimated for such times (fig 3) The differences, d, between the actual values of B or b and these extrapolated values may be obtained by subtraction It has been shown (DOST 1968) that the natural logarithm of this difference, $\ln D = \ln (B' - B)$ or $\ln d = \ln (b' - b)$, where $b = B/V_f$) will have real values until the drug is completely absorbed and plots of these functions against time will give a new straight line of the equation

$$\ln d = -k_a t + \ln b_o \qquad \text{(Eq 8)}$$

and the apparent first order rate constant of absorption, k_a, can be estimated from its slope This method is called "feathering the curve In many European languages, the term "peeling the apple" is used to describe this process

If the logarithm of this difference against time is not linear, the absorption may not be stimulated by the assumption that it is a first order process However, it is surprising how often such a first order process serves to characterize the absorption process in practice

The determination of the rate constants, k_a and k_e, by the above specified methods permits the estimation of the blood curve at any time from the expression

$$b = \frac{B}{V_f} = \frac{\gamma D_o}{V_f} \frac{k_a}{(k_e - k_a)} (e^{-k_a t} - e^{-k_e t}) \qquad \text{(Eq 9)}$$

when the time duration of a blood level b above a certain desired minimum b_{min} from the administration of a solution of drug, D_o, can be calculated If the rate of release of the drug from the formulation is the rate determining factor in the absorption then this calculated interval of a blood level above a b_{min} value can serve in the estimation of biological availability of a drug dosage D_o from various formulations

Other criteria of biological availability which would fully characterize drug-time appearance curves in the blood on the basis of a sequential first order model are the maximum blood level, b_{max}, achieved and the time, t_{max}, at which this maximum is reached

These values are related to the first order absorption, k_a, and elimination constants, k_e, by the following expressions

$$b_{max} = (A_o/V_f)(k_e/k_a)^{k_e/(k_a - k_e)} \qquad \text{(Eq 10)}$$
$$t_{max} = (\ln k_a/k_e)/(k_a - k_e) \qquad \text{(Eq 11)}$$

If both the rate of absorption and the rate of release from the dosage

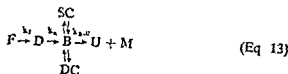
form are characterized by first order rate constants, i e k_a and k_f in Eq 7 respectively, then a further "feathering" of the blood level time curves permit estimates of the apparent first order rate constant, k_f, for the *in vitro* release of the drug from the formulation when $k_a < k_f$ Under these conditions, the plot of logarithm d against time will not be linear during the initial time after administration of the formulation If the terminal segment of the ln d *versus* time curve is linear, it permits estimation of the apparent absorption rate constant, k_a, from the slope in accordance with Eq 8 and extrapolation of this linear segment to times when the drug is being released from the formulation Thus, values of d' can be estimated from such times The differences f, between the actual values of d and these extrapolated values, d', may be obtained by subtraction *It has been shown* (DOST 1968) *that the natural logarithm* of this difference ln f = ln (d' − d) will have real values *until the drug is completely* released from the formulation *in vivo* Plots of these functions against time will give a new straight line and the apparent first order rate constant of formulation release, k_f, can be estimated from its slope In practice, practically all formulation release rates (except perhaps from a mass of coated pellets of different thickness of coating (GARRETT & LAMBERT 1966) are apparently first order

If $k_a \gg k_f$, which is most frequently the case, Eqs 8–11 hold except that k_f may be substituted for k_a, and the single feathering is all that is needed Thus, the blood level as a function of time may be postulated to conform to Eq 9 where the k_a is now k_f The b_{max} and t_{max} values of Eqs *10 and 11 may be* considered as valid parameters of release from the *formulation*

The *model of Eq* 7 *may be* further complicated (DOST 1968) by a significantly time-dependent *first order redistribution* of the amount of drug actually in the blood compartment, B' *into other tissues, generally summarizable* into an amount in a deep, DC, and shallow, *SC, compartment* so that the B of Eq 7 is actually

$$B = B' + DC + SC \qquad \text{(Eq 12)}$$

and



$$F \xrightarrow{k_f} D \xrightarrow{k_a} B \xrightarrow{k_{B,U}} U + M \qquad \text{(Eq 13)}$$

(with B ⇅ SC above and B ⇅ DC below)

Only when k_a and/or k_f are *relatively slow* and k_{BU} *is relatively* fast with respect to these SC and DC *equilibrations, is it necessary* to propose and use the more complicated model *of Eq 13 rather than that of* Eq 7 Under such circumstances, the best evaluations *of the backwards* and

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form are characterized by first order rate constants, i.e. k_e and k_f in Eq. 7 respectively, then a further "feathering" of the blood level-time curves permits estimates of the apparent first order rate constant, k_f, for the *in vitro* release of the drug from the formulation when $k_a < k_f$. Under these conditions, the plot of logarithm d against time will not be linear during the initial time after administration of the formulation. If the terminal segment *of the ln d versus time curve is linear, it permits estimation of the apparent absorption rate constant,* k_a, from the slope in accordance with Eq. 8 and extrapolation of this linear segment *to times when the drug is being re*leased from the formulation. Thus, values *of d′ can be estimated from* such times. The differences, *f*, between the actual values of d and these extrapo [illegible] be obtained by subtraction. It has been shown (Dost [illegible] − d) will ha [illegible] ormulation *in vivo*. Plots of these functions against time will give a new straight line and the apparent first order rate constant of formulation release, k_f, can be estimated from its slope. In practice, practically all formulation *release rates* (except perhaps from a mass of coated pellets of different thickness of coating (Garrett & Lambert 1966) are apparently first order.

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and

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Only when k_a and/or k_f are relatively *slow and* k_{BU} is relatively fast with respect to these SC and DC equilibrations, is it necessary to propose and use the more complicated model of Eq. 13 rather than that of Eq. 7. Under such circumstances, the best evaluations of the backwards and

forwards rates constants implicit in Eq 13 are obtained from pharmaco kinetic studies after rapid intravenous injection

Other methods of estimation of absorption when only urinary excretion is known

If only data on drug (or metabolite derived from drug) excreted in the urine is available, the ability to fit models is severely limited The only reasonable model to assume is that given by Eq 7 with its previously stated underlying assumptions NELSON (1959, 1960 & 1961) derived an expression based on this model and the stoichiometry

$$\gamma D_o = (F + D) + B + (M + U) \qquad \text{(Eq 14)}$$

where the absorbed fraction γ_o of the dose D_o absorbed is distributed as the amount B in the apparent volume of distribution, as the amount, U + M, eliminated or transformed, and as the amount, F + D, still at the absorption site The factors of Eq 4 can be derived with respect to time and

$$-d(F + D)/dt = dB/dt + d(U + M)/dt \qquad \text{(Eq 15)}$$

i e, the rate of absorption is equal to the sum of the rates of change of the amounts of drug B in the blood and the amounts, U + M, excreted and transformed

The last term of the equation is (GARRETT 1971)

$$d(U + M)/dt = dU/dt + dM/dt \qquad \text{(Eq 16)}$$
$$= (k_{B\rightarrow U} + k_{B\rightarrow M})B$$

and $dU/dt = k_{B\rightarrow}$ [illegible] $_{\rightarrow M})fB$ (Eq 17)

where f is the fraction of the drug excreted into the urine (23 25) so that

$$d(U + M)/dt = (dU/dt)/f \qquad \text{(Eq 18)}$$

and

$$d^2U/dt^2 = (k_{B\rightarrow U} + k_{B\rightarrow M})f(dB/dt) \qquad \text{(Eq 19)}$$

When factors from Eq 18 and 19 are substituted into Eq 15 and k is taken as $k_{B\rightarrow U} + k_{B\rightarrow M}$, the absorption rate can be expressed as

$$dA/dt \approx -d(F + D/dt = d^2U/dt^2/k_e f + (dU/dt)/f \qquad \text{(Eq 20)}$$

Thus, if k_e can be obtained from the slope of the linear plot of the

terminal data of ln $(U_\infty - U)$ against time, i e , after absorption is completed, and if f, the total fraction of absorbed drug that is renally excreted unchanged, is known, all that is necessary to obtain the instantaneous rate of absorption in Eq 20 is the instantaneous first and second derivatives of the urinary excretion rates of the unchanged drug The first derivative, dU/dt, is obtainable at any given time from a) the instantaneous slopes or tangents to the curve of total accumulated drug excreted in the urine against time or b) from the amount of drug excreted in a given interval of time. The second derivative, d^2U/dt^2, can be obtained from the tangents to the plot of the instantaneous rates of urinary excretion against time This method has many limitations The major one is the *difficulty of obtaining accurate urinary excretion data and sufficient numbers of urinary analyses during the drug absorption phase* The taking of second derivatives by such a process is also a risky business

Eq 20 can be integrated (WAGNER & NELSON 1963 & 1964) so that the amount, A_t of drug absorbed up to time t can be expressed as

$$A_t = [(dU/dt)/k_e + U]/f \qquad \text{(Eq 21)}$$

Thus, from the knowledge of the excretion rate, dU/dt, at any time, i e , the amount excreted per unit time, and the total cumulative amount of drug, U, excreted into the urine up to that time, the amount of drug, A_t, absorbed at any given time can be calculated This Eq 21 needs no assumption of order of absorption or of drug release since it determines the overall transfer of F and D to the body, B, (Eq 7) by any process whatsoever at any time, t.

If the same individual is used to compare two different formulations so that the fraction, f, of drug, excreted unchanged can be assumed to be constant fA_t values can be calculated from Eq 21 without explicit knowledge of f Thus, the ratios of these values give the relative amounts absorbed, $(A_t)_1/(A_t)_2$, from formulations 1 and 2 at any given time

Other methods of estimation of absorption when both urinary excretion and blood level time curves are known

The substitution of the rearranged Eq 19 for dB/dt into eq 15 is not necessary when the blood level time data is also known and the instantaneous rate of absorption could then be defined as

$$dA/dt = -d(F + D)/dt = V_f(db/dt) + (dU/dt)/f \qquad \text{(Eq 22)}$$

where $b = B/V_f$ and db/dt can be obtained from the instantaneous slope of the concentrations of drug in the blood, i e , b, against time

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If the same individual is used to compare two different formulations so that V_f can be assumed to be constant, A_t/V_f values can be calculated from Eq 26 without explicit knowledge of V_f. Thus, the ratios of these values give the *relative amounts* absorbed, $(A_t)_1/(A_t)_2$ from formulations 1 and 2 at any given time

These several methods are limited in that they presume first order removal of drug from the body into the excreta and metabolites, that *apparent first order* rate constants of equilibration, elimination and metabolism are independent of blood level and dose, that there is no storage in an intermediate compartment or lag between the loss from the blood and the appearance in the monitored excreta

Solubilization as a determining factor in drug availability

If the drug is to be administered for reasons of pharmaceutical elegance or stability in a solid dosage form, it must go into solution at adequate rates so as to present itself in its proper diffusional regalia at the biological membrane These rates of dissolution increase with decreasing particle size and increasing surface area The higher the solubility and agitation, the greater the rate of dissolution in an isotropic medium The formal shape of the drug particle will have a definitive effect on the time course of dissolution when surface area is important

The Noyes-Whitney law (NOYES & WHITNEY 1897) describes the rate of dissolution of an amount, W, of solid by a diffusion-controlled process

$$\text{Rate of dissolution} = dW/dt = D''S\,(C_s - C)/h \qquad \text{(Eq 27)}$$

where h is the thickness of the diffusion layer, S is the surface area of the solid exposed C_s is the concentration of a saturated solution, C is the concentration of drug in the body of the solution and D'' is a constant The factor h will vary with the intensity of agitation This basic model considers the rate determining step to be diffusion of the drug from a layer of saturated solution in contact with the solid interface to the body of the solution.

Variations of this law have been applied to dissolution of crystals and solids in which the geometry of the dissolving material and the change of S with time and degree of dissolution have been considered (HIXSON & CROWELL 1931, PARROTT *et al* 1955, WURSTER & SEITZ 1960)

At high agitations diffusion control is diminished and the tendency is for the dissolution rate to be controlled by the rate of release of drug from the crystal or the rate of solvation of the molecule from the crystalline matrix (WURSTER & TAYLOR 1965) This rate of release would be independent of viscosity and have a higher apparent heat of activation than

An approximate estimate of V_f may be obtained from the zero time intercept, i e ln b_o, of the ln b versus time plot where $V_f = \gamma D / b_o$ where γD_o is the amount of drug absorbed An alternative method of obtaining V_f is from the renal clearance After completion of the absorption phase, the model of Eq 7 states that the rate of urinary excretion is

$$dU/dt = k_{B \rightarrow U} B = k_e f V_f b \qquad \text{(Eq 23)}$$

Thus, a plot of the instantaneous rate of urinary excretion of the drug against the blood level at that time will give the renal clearance, $k_e fV_f$ as the slope Since k_e and f can be readily obtained by the methods previously described, V_f can be estimated

Integration of Eq 22 results in the amount absorbed, A_t, at any time, t

$$A_t = V_f b + U/f \qquad \text{(Eq 24)}$$

when b is the concentration in the blood and U is the amount of drug excreted as such into the urine at that time

Other methods of estimation of absorption when only blood level data are known

Since Eq 23 can hold for all time intervals it can be substituted into Eq 22 to give the rate of absorption as

$$dA/dt = -d(F + D)/dt = V_f(db/dt + k_e b) \qquad \text{(Eq 25)}$$

Thus, the determination of blood level, b, and its rate of change at a particular time permits the estimation of the instantaneous absorption rate Obviously, when $-db/dt = k_e b$, $dA/dt = 0$ or conversely

Integration of Eq 25 results (WAGNER & NELSON 1963 & 1964) in the amount absorbed A_t, at any time, t

$$A_t = V_f \left(b + k_e \int_0^t b\,dt\right) \qquad \text{(Eq 26)}$$

Thus, the area under the drug concentration in the blood time curve up to a time, t, i e, $\int_0^t b\,dt$, and knowledge of the blood level b at that time permit the estimation of the amount absorbed up to that time This Eq 26 needs no assumption of order of absorption or drug release since it determines the overall transfer of F and D to the body B (Eq 7) by any process whatsoever at time t

be completely independent of their disintegration times (BRUDNEY *et al* 1964) but the dissolution of tolbutamide tablets was correlated with clinical efficacy (LEVY 1964)

It has been shown that the absorption of the slightly water soluble antibiotic griseofulvin is rate limited by the solution process and that the particle size of the administered drug effects the availability of this drug for absorption (RIEGELMAN 1962)

The biological activities of different salts of benzphetamine were shown to be definitely dependent on their dissolution rates (MOROZOWICH *et al* 1962) Plasma levels of various penicillins were correlated with their solubilities (JUNCHER & RAASCHOW 1957)

Clinically ineffective tablets of prednisone were shown to have lower dissolution rates than those that were clinically effective although U S P disintegration tests were passed and labelled drug content was present (CAMPAGNA *et al* 1963, LEVY *et al* 1964) Conflicting clinical reports concerning the relative advantages of plain and buffered aspirin tablets have been ascribed to the observed differences in dissolution rates among various products (LEVY & HAYES 1960) As indicated by estimation of blood plasma levels of sulfadiazine, highly significant differences in the biological availability of orally administered drug were observed Absorption was most rapid when the drug was given as micro crystals, next most rapidly as the micronized powder and least rapidly as the tablets (BOYD & DINGVALL 1947, REINHOLD *et al* 1945)

Solubilization was again implicated when the biological availability of tablets of spironolactone was greatly increased by preparing the active ingredient in the form of a fine powder in hard gelatin capsules or by dispersing the drug in tablets with a larger volume of water-soluble matrix (LEVY 1962)

The release of dicumarol from tablets in a very finely divided form necessitated a complete re titration of patients on anticoagulant therapy since the rate of solubilization was enormously enhanced (LOZINSKI 1960)

Evidence for the importance of pharmaceutical adjuvants and excipients as they affect physical factors

Since solubility can be considered as a tug-of war between the energetics of solvation and crystalline forces, one way is to make a complex or solid solution of the drug so that molecules of the drug are more readily solvated than they would be in the crystalline matrix There has been little reliable evidence that excipients can promote absorption on any other ground than that they compete for compounds that may complex the drug and make it unavailable or that they promote disintegration of the solid

diffusion-controlled dissolution rates When diffusion control is important, i e, when the rate determining step is the transport of the solute from the interfacial boundary to the bulk of the surrounding solution, the viscosity of the fluid is important since diffusion constants are inversely proportional to viscosity, e g the Stokes-Einstein equation (WURSTER & TAYLOR 1965)

The thermodynamic stability of the crystalline form is also vital (HIGUCHI *et al* 1963) An unstable crystalline polymorph is preferred for the more rapid dissolution Amorphous novobiocin can be solubilized, the antibiotic is unavailable for gastrointestinal absorption from the beautifully crystalline form (MULLINS & MACEK 1960) Metastable polymorphs have higher solubilities and hence faster dissolution rates than stable forms of the same compound (HIGUCHI 1958)

In general, salts will show higher dissolution rates than the corresponding nonionic drug at any pH even though the final equilibrium solubility of the drug and its salt would be the same (HIGUCHI *et al* 1958) This is readily explained by the fact that if precipitation occurs, a suspension of fine particles result which have the proper characteristics for proper redissolution An accepted fact is that *in vitro* dissolution tests must be correlated with *in vivo* estimates of physiological availability (WAGNER 1961), but they may serve as estimates of quality control

In some instances high concentrations of dissolving drugs or compounds surrounding particles can irritate the intestinal mucosa A classical example is the recent trouble with solid KCl in diuretic preparations Since true solutions of equivalent amounts of KCl (such as in natural Florida orange juice) were prefectly safe and non-toxic, a preparation that produces small and rapidly dissolving particles was necessary for proper formulation A precursor molecule for a drug, or a pro-drug, may have to be solvolyzed before absorption, e g, chloramphenicol palmitate (GLAZKO *et al* 1952) Again, dissolution must precede this solvolysis and is dependent on particle size Any phenomena which will abet the solubilization of the active ingredient are right and proper

Evidence for the importance of dissolution rates and solubilities

Crystalline preparations of palmitate and stearate esters of cloramphenicol produced low blood levels whereas finely divided suspensions produced high blood levels This phenomenon was ascribed to the necessity of solvolysis prior to absorption which demands a rate of dissolution dependent on the smallness of particle size for best overall absorption rates (GLAZKO *et al* 1952 & 1958)

The polymorphic form of chloramphenicol palmitate significantly influenced its solubility and thus the blood levels obtained (AGUIAR *et al* 1967) The dissolution rates of many tolbutamide tablets were shown to

ultimate criterion is biological availability although *in vitro* tests may give proper evidence and correlation

There have been many machines proposed to give amounts of drug dissolved from a formulation in a solvent as a function of time. At the present time, one is even given in the most recent edition of the U.S.P. The choice of a universal apparatus or machine to be used in *effecting standardized* dissolution studies as a method of *quality control is highly controversial* at the moment. However, *the use of dissolution rates is accepted* as valid *but the argument has* been made that the apparatus used and the conditions under which it is used may have to be varied from preparation to preparation

The apparent consensus of opinion is that such an apparatus must provide agitation that is sufficient to permit a changing flow of fluid on the surfaces of the preparation (tablet, capsule, etc.) so that any disintegration is primarily the result of solvation rather than physical disruption by force of collision with the walls of the container. The agitation must be sufficient to disperse the solvated particles *and provide a homogeneous solution* of dissolved drug without *physically rupturing the dispersed particles* The agitation must not be so excessive that its strips off the saturated layer in contact with the surfaces of the dissolving particles so that the

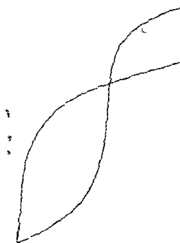


Fig. 4 Example of types of dissolution curves that can be obtained for two *different* formulations of the same drug. A formulation (1) may show a faster rate of absorption of the initial 50 % *of the drug* than formulation (2) whereas the last 50 % or less of a formulation (2) may be much more readily *dissolved than* that of formulation (1) One formulation, e.g. (1), may show little or no lag or induction *phase prior to significant dissolution* whereas another formulation, e.g. (2), may show a highly significant lag phase

dosage form or promote its solubilization (the 'buffer" aspects of buffered aspirin) (WAGNER 1961) These are not unimportant since tablet *disintegration* is a necessary preliminary step to tablet dissolution, but not necessarily a major factor in the overall process of absorption Excipients may interact with and diminish the availability of the drug by promoting instability, or form a derived molecule which is inactive and unabsorbable Coatings may age on storage and change the release characteristics of the formulation These physical and chemical factors are of enormous importance in proper formulation design

Many so-called inert adjuvants and excipients are not truly *inert* Polyethylene glycol 4000 forms a complex with phenobarbital and reduces its solubility The net result is that the dissolution and subsequent intestinal absorption rates were reduced (SINGH *et al* 1966) Polysorbate 80 has considerable influence on the stability and availability of ionic drugs *in pharmaceutical* formulations Such drugs are cations such as chlorpromazine, promethazine, tetracaine, methylrosaniline and dodecyl pyridinium, and anions such as napthalene sulfonate and methyl orange (HURWITZ *et al* 1963)

The insoluble complex of amphetamine and carboxymethylcellulose inhibits biological availability (WAGNER 1964) Calcium excipients such as dicalcium phosphate decrease the serum concentrations of administered tetracycline through the probable chelation of calcium with tetracycline, and lower tetracycline absorption from the gastro-intestinal tract (BOGER & GAVIN 1959)

The lack of biological availability and erratic *in vivo* appearance of aspirin and a thyroid medication from enteric coated tablets has been ascribed to the resistance of the coating to dissolution (CLARK & LASAGNA 1965, CORBUS 1964)

Excellent detailed reviews and critiques on these themes are available The papers of NELSON (1961) on drug absorption, distribution, metabolism and excretion and WAGNER (1961 & 1964) on the absorption aspects of biopharmaceutics are comprehensive Tablet disintegration and physiological availability have been considered by MORRISON & CAMPBELL (1965) whereas WURSTER & TAYLOR (1965) have considered dissolution rates and availability LEVY & NELSON (1961a & b) have cited many clinical examples where these physico chemical factors have affected drug availability and reliability of response

In vitro dissolution rates and their interpretation

The importance of these physico-chemical factors of drugs and drug systems in affecting the availability and reliability of response can be substantiated by the cited data and observations in the literature The

ultimate criterion is *biological availability although in vitro* tests may give proper evidence and correlation

There have been many machines proposed *to give amounts of drug* dissolved from a formulation in a solvent as a function of time At the present time, one is even given in the most recent edition of the U S P The choice of a universal apparatus or machine to be used in effecting standardized dissolution studies as a method of quality control is highly controversial *at the moment However, the use of dissolution rates is accepted* as valid but the argument has been made that the apparatus used and the conditions under which it is used may have to be varied *from preparation* to preparation

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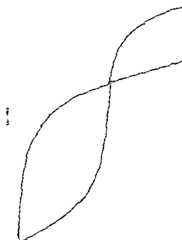


Fig 4 Example of types of dissolution curves that can be obtained for two different formulations of the same drug. A formulation (1) may show a faster rate *of absorption of the initial 50 %* of the drug than formulation (2) whereas the last 50 % or less of a formulation (2) may be much more readily dissolved than that of formulation (1) One formulation e g (1) may show little or no lag or induction phase prior to significant dissolution whereas another formulation e g (2), may show a highly significant lag phase

dissolution rate becomes thermodynamically controlled by solvation and release from the crystalline matrix rather than by diffusion from this layer into the bulk of the solution This latter process must be maintained as the rate determining step to give the greatest sensitivity in differentiating among the dissolution rates of the several preparations that are to be compared

The plots of amount of drug in solution against time for a fixed rate and kind of agitation for a fixed geometry of the dissolution vessel and for a fixed volume of solvent are the resultant experimental data (fig 4) The solvents used should simulate those biological fluids in which the preparation will dissolve and thus should be limited to artificial gastric juice, 0 1 N-HCl, or phosphate buffers of specified pH in the 5–7 pH range An organic or mixed organicaqueous solvent should not be used since dissolution rates in such media *do not* necessarily parallel those of purely aqueous biological media The fact that dissolution rates are related to the solubility of the drug in the solvent (Eq 27) should make this quite apparent

If the components of a tablet or capsule were dispersed in a solvent, the crystals of the active ingredient will decrease in size with the process of dissolution and the concentration, C, of the bulk solution would increase with time If the change in particle size were ignored, the rate of dissolution with respect to the Noyes-Whitney law of Eq 27 would reduce to

$$dW/dt = K(C_S - C) \qquad \text{(Eq 28)}$$

and would decrease with time as the concentration of the drug, C, in the bulk solution approaches the solubility, C_S, of the drug in the solvent At constant volume of solvent, the amount dissolved, W, is proportional to C and integration of Eq 28 results in an expression of the form

$$\ln (C_S - C) = -K't + \ln C_S \qquad \text{(Eq 29)}$$

and plots of the logarithm of the difference between the concentration in the bulk solution and the solubility should be linear against time and the apparent negative slope, K′, could be considered as a proper criterion of solubilization and dissolution

If the concentration of the bulk solution is kept sufficiently small so that C approaches zero, or if the drug in the bulk solution is instaneously removed from the solution as by partitioning into an organic solvent, "sink" conditions are assumed to exist If Eq 28 is valid, plots of amounts dissolved (or concentrations in the organic phase) against time should be linear and the slopes of such plots may be considered as estimates of dissolution rates

A frequent improper application of the assumptions underlying Eqs 28 or 29 is to plot the logarithm of the difference between the concentration, C of drug in the bulk solution and the concentration, C_T, after all the drug has dissolved. There is no proper rationale for this treatment of the data since normal and reasonable experimental conditions demand that C_T should be significantly less than C_s In addition, the implicit assumption for the use of Eq 29, that there is no significant decrease in particle size or total surface area throughout *the dissolution process, only approaches validity when the dispersed particles of released drug are extremely small and numerous*

Nevertheless, the plots of the logarithms of $C_S - C$ or $C_T - C$ against *time for a fixed volume of chosen solvent*, with or without "sink" conditions, occasionally give curves that can be fitted by a straight line Then the empirical use of apparent K′ values (negative slopes of expressions similar to Eq 29) may be operationally useful as comparative dissolution rate parameters More frequently, such semi logarithmic plots of $C_s - C$ or $C_T - C$ are non linear but can be fitted to a linear sum of exponentials by the previously described "feathering" techniques so that

$$C = C_S - A_1 e^{-k_1 t} - A_2 e^{-k_2 t} \cdots - A_{n-1} e^{-k_{n-1} t} - A_n e^{-k_n t} \quad \text{(Eq 30)}$$

where the slope of the linear *terminal* $\ln(C_S - C)$ *versus t data estimates* k_n with an apparent intercept of A_n Subsequent feathering estimates a new slope k_{n-1} and intercept A_{n-1} from the newly generated terminal data etc This expression implicitly states that

$$C_S = A_1 + A_2 + \cdots + A_{n-1} + A_n \quad \text{(Eq 31)}$$

where $C = 0$ at time zero and $C = C_S$ at infinite time If the plots were derived from $\ln(C_T - C)$data, then C would be expected to approach the C_T value at *infinite time The parameters of Eq* 30 are susceptible to interesting interpretations For $C_T - C$ data, the $A_1/C_T, A_2/C_T, \cdots A_{n-1}/C_T$, A_n/C_T fractions of *the amount of drug in the formulation* may be considered as different populations that dissolve with apparent respective first order rate constants of $k_1, k_2, \cdots k_{n-1}$, and k_n An equation similar to Eq 30 is also applicable when there is a lag period for dissolution due to a time dependent disintegration process Under such circumstances, one or more of the coefficients of exponential terms may be positive Digital computer fitting for non linear regressions also can give best estimates of the parameters for a linear sum of exponentials to describe the increasing concentration of drug in the dissolution media

An alternative method is to fit C versus t data by non-linear regression techniques to a polynomial such as

$$C = a + bt + ct^2 + \ldots + zt^n \qquad \text{(Eq 32)}$$

This can be most probably limited to a three or four term polynomial There are two major criticisms of this method. The one is that such an expression as Eq 32 may fit all the data points given but not be valid for interpolated values The other is that the values of a,b,c, . . and z do not have any rational significance for comparisons of formulations This is in contrast to the Eq 30 which permits comparisons of relative amounts of drug, i e, A_0, in reasonably similar apparent first order dissolution rate populations, i e, with approximately similar values of k_1

Since delays in disintegration of, or release from a matrix in a formulation may give an S shaped curve of C versus t (fig 4), a logit type transformation may occasionally give a linear plot for all the data Thus a plot of $\log \frac{C/C_T}{1 - C/C_T}$ versus t or log t may linearize the data so that the intercept and slope may serve to characterize the dissolution rates in accordance with an equation such as

$$\log \frac{C/C_T}{1 - C/C_T} = a \log t + b \qquad \text{(Eq 33)}$$

Plotting on probit or logit paper may be done directly to check such linearizations If such linear transformations work for several formulations of a drug, they would be operationally useful, but the opinion can be expressed that it would be fortuitous since it would only be due to fortunate compensations of imbalances in the C versus t curve

The dissolution rate curves of C versus t are complex They are the products of many potential interacting phenomena which can include a) fracture of a coating at its weakest spot after weakening by solvent and subsequent leaching of drug from that spot, b) permeation of solvent into the mass of the formulation matrix, c) swelling and gummy mass formation that may inhibit permeation, d) disintegration of the formulation mass into cohered masses, particles and/or crystals and e) solvation and dissolution of crystalline particles Thus, fanciful fitting of the drug concentration versus time data from dissolution rate studies may be an excercise in futility. Such fittings, as by either Eq 29, 30, 32 or 33 must be consistent for all formulations to permit valid comparison and this is usually not the case. Thus, comparisons of tabulations of times that specified amounts of drug are released to the solution may be even more valid

estimates of dissolution rates than *those* derived from elegant curve fitting

Suggested values are *times* for 25, 50, 75, 85 and 95 % dissolutions *It is perfectly* possible for one formulation to release *50 % in 5 minutes* and take 5 hours to release the last 50 % (or 5 %) Is this a better formulation than one that releases 50 % in 30 minutes, but only takes 1 hour to release the last 50 % (or 5 %)? (See fig 4)

Dissolution rate studies may be used *to* compare prospective formulations and the release rates of drug from them The conditions of agitation, solvent, etc. will obviously be arbitrary *with the hope that they will reflect* differences in potential biological availabilities

It is certainly rational to choose a preferred formulation on these bases However, it is *not* proper to assume that differences in apparent *in vitro* dissolution rate among formulations will be significant in an *in vivo* situation. Also, if there are *no differences in in vitro* dissolution rates among formulations in the arbitrary system chosen, it is *not* proper to assume that there will be no difference in the *in vivo* situation Biological availability must be correlated with *in vitro* dissolution rates to substantiate the validity of the latter Of course, dissolution rates under fixed conditions may serve as proper quality control procedures for accepted formulations Certainly, a process *change or an aging phenomena that changes such* dissolution rates may change biological availabilities and thus such *in vitro* techniques may serve as a screen for potential changes in *in vivo* availability

REFERENCES

Aguiar A. J., J Krc, A. W Kinkel & J C. Samyn *J Pharm Sci.* 1967 *56*, 847

Boger W P & I I Gavin *New England J Med.* 1959, *261*, 827

Boyd E M & R. W Dingwall *Amer J Med Sci* 1947, *213*, 549

Brodie B B In *Absorption and distribution of drugs* Ed T B Binns. E & S Livingston Ltd., London 1964

Brodie B B & C. A. M Hogben *J Pharm* (London) 1957, *9*, 345

Brudney N D J Stewart & B T Eustace *Can Med Ass J 1964* *90*, 980

Caminetsky S *Can. Med Ass J* 1963, 88 950

Campagna, F A., G Cureton, R. A. Mirigian & E. Nelson *J Pharm Sci* 1963, *52*, 605

Cannell J S *J Pharm Pharmacol.* 1951, *3*, 741

Carter A K *Can Med Ass J* 1963 88 98

Castello R. A & A. M Mattocks *J Pharm Sci* 1962, *51*, 106

Chapman, D G., R. Crisafio & J A. Campbell *J Amer Pharm Ass*, *Sci Ed* 1954, 43 297

Clark, R L. & L. Lasagna *Clin. Pharmacol Therap* 1965, *6*, 568

Corbus, H. P *Calif Med* 1964, *100*, 364

Dost, F H *Grundlagen der Pharmakokinetik* 2nd ed G Thieme Verlag Stuttgart, 1968.

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Schanker, L. S, P A Shore, B B Brodie & C. A. M Hogben *J Pharmacol* 1957, **120**, 528
Schanker, L. S, D J Tocco, B B Brodie & C. A. M Hogben *J Pharmacol* 1958, **123**, 81
Schroeter, L. C, T Higuchi & E. E Schuler *J Amer Pharm Ass, Sci. Ed* 1958, **47**, 723
Shore, P A, B B Brodie & C A M Hogben *J Pharmacol* 1957, **119**, 361
Singh, P., J K. Guillory, T D Sokoloski, L. Z. Benet & V N Bhatia *J Pharm Sci* 1966 **55**, 63
Troup, A. E. & H Mitchner *J Pharm Sci* 1964, **53**, 375
Wagner, J G *J Pharm Sci* 1961, **50**, 359
Wagner, J G *Antibiot Chemotherapy (Basel)* 1964, **12**, 53
Wagner, J & E Nelson *J Pharm Sci* 1963, **52**, 610
Wagner J & E. Nelson *J Pharm Sci* 1964, **53**, 1392.
Wurster, D E. & J A. Seitz *J Amer Pharm Ass, Sci. Ed* 1960, **49**, 335
Wurster, D E & P W Taylor *J Pharm Sci* 1965, **54**, 169

Garrett, E R *J Amer Pharm Ass, Sci Ed* 1960, **49**, 767
Garrett, E. R *J Pharm Sci* 1962, **51**, 410
Garrett E R Kinetics and mechanisms in stability of drugs In *Advances in pharmaceutical sciences* Vol II, sphater I Academic Press 1967
Garrett, E R Theoretische pharmakokinetik *In Klinische Pharmakologie und Pharmakotherapie* Ed H P Kuemmerle, E R Garrett & K H Spitzy Urban und Schwarzenberg, Munich 1971, p 27
Garrett, E R, S Goto & J F Stubbins *J Pharm Sci* 1965, **54**, 119
Garrett, E R & H J Lambert *J Pharm Sci* 1966, **55**, 626
Garrett, E R & D J Weber *J Pharm Sci* 1962, **51**, 387
Glazko, A J, W Edgerton, W Dill & W Lenz *Antibiot Chemotherapy* 1952, **2**, 234
Glazko, A J, W Dill, A Kazenko, L M Wolf & H E Carnes *Antibiot Chemotherapy* 1958, **8**, 516
Higuchi, T *J Amer Pharm Ass Sci Ed* 1958, **47**, 657
Higuchi, W I, P K Lau, T Higuchi & J W Shell *J Pharm Sci* 1963, **52**, 150
Higuchi, W I, E L Parrott, D E Wurster & T Higuchi *J Amer Pharm Ass, Sci Ed* 1958, **47**, 376
Hixson A W & J H Crowell *Ind Eng Chem* 1931, **23**, 923
Hogben, C A M L S Schanker, D J Tocco & B B Brodie *J Pharmacol* 1957, **120**, 540
Hobben, C A M, D J Tocco, B B Brodie & L S Schanker *J Pharmacol* 1959, **125**, 275
Hurwitz, A R, P P DeLuca & H P Kostenbauder *J Pharm Sci* 1963, **52**, 893
Juncher, H & F Raaschow *A M & C T* 1957, **4**, 497
Levy, G *Lancet* 1962, 723
Levy, G *Canad Med Ass J* 1964, **90**, 978
Levy G, N A Hall & E Nelson *Amer J Hosp Pharm* 1964, **21**, 402
Levy, G & B A Hayes *New England J of Med* 1960, **262**, 1053
Levy, G & E Nelson *J Amer Med Ass* 1961a **117**, 689
Levy G & E Nelson *N Y J of Med* 1961b, **61**, 4003
Lozinski, E *Canad Med Ass J* 1960, **83**, 177
Morozowich, W, T Chulski, W E Hamlin, P M Jones, J L Northam, A Purmalis & J G Wagner *J Pharm Sci* 1962, **51**, 993
Morrison, A B & J A Campbell *J Pharm Sci* 1965, **54**, 1
Mullins, J D & T J Macek *J Amer Pharm Ass, Sci Ed* 1960, **49**, 245
Nelson, E *J Pharm Sci* 1959, **48**, 489
Nelson E *J Pharm Sci* 1960, **49**, 437
Nelson, E *J Pharm Sci* 1961, **50**, 181
Noyes, A A & W R Whitney *J Amer Chem Soc* 1897, **19**, 930
Parrott, E L., D E Wurster & T Tiguchi *J Amer Pharm Ass, Sci Ed* 1955, **44** 269
Reinhold, J G, F J Phillips & H F Flippin *Amer J Med Sci* 1945, **210**, 141
Ribeiro, D, D Stevenson, J Samyn, G Milosovick & A M Mattocks *J Amer Pharm Ass Sci Ed* 1955, **44**, 226
Riegelman, S, W L Epstein & R I Dayan Paper B IX, Preprints of papers (Scientific section of American Pharmaceutical Association Las Vegas 1962)
Schanker, L S *J Pharmacol* 1959, **126**, 283
Schanker, L S *J Med Pharm Chem* 1960, **2**, 343
Schanker, L S *Ann Rev Pharmacol* 1961, **1**, 29
Schanker, L S *Pharmacol Rev* 1962, **14**, 501

Schanker, L S, P A. Shore, B B Brodie & C. A. M Hogben *J Pharmacol* 1957, **120**, 528
Schanker, L. S, D J Tocco, B B Brodie & C. A M Hogben *J Pharmacol* 1958, **123**, 81
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Physico-Chemical Factors of Drugs Affecting Absorption, Distribution, and Excretion

By

Heinrich Keberle

Key words Physico chemical properties of drugs – absorption – distribution

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This paper, as indicated by its title, deals with the influence which some physico-chemical properties of drugs exert upon their behaviour in the organism The physico-chemical properties that are relevant to absorption will be described first A list of the main factors is given in table 1

The importance of these factors – lipid and water solubility, degree of ionisation, chemical stability of the drug, and molecular weight – is already very well known and has frequently been emphasised It is agreed that a substance will be well absorbed if it is liposoluble and at the same time also soluble to some extent in water, if it is not completely ionised, if it is not too unstable in the lumen of the gastro-intestinal tract, and finally, if its molecular weight is not excessively high

It must be pointed out, however, that this general type of description of the physico-chemical factors which should facilitate absorption, does not prove of much use in practice until one has defined more closely the scope and significance of the individual factors To illustrate this, the examples to be cited will not be ones confirming the rules just mentioned – because plenty of these can be found in the literature – but examples which appear at first sight to contradict the rules In this connection, reference will also be made to the difficulties encountered when measuring the physico chemical properties of a drug

The examples to be quoted have all been taken from our own experience

Listed in table 2 are data on the solubility and distribution of two drugs The first is an orally active muscle relaxant, and the second has amoebicidal activity As can be seen from the table, the first compound is moderately soluble in water, the respective figure being 400 mg/l In butanol octanol, and methylene chloride it is only very sparingly soluble, whilst in ether, benzene, and hexane it is practically insoluble Upon partition of the

Table 1

Physico-chemical properties affecting the absorption of a compound.

1	Lipid solubility
2	Water solubility
3	Degree of ionisation
4	Chemical stability
5	Molecular weight

compound between water and the same volumes of organic solvent, 40 % dissolved in butanol, 10 % in octanol, only 0 3 % in methylene chloride, and virtually nothing in the ether, benzene, or hexane phase Judged by the usual criteria this substance displays moderate water solubility, whereas its liposolubility is extremely slight or practically nil This would seem to suggest that its enteral absorption must be minimal

An examination of the corresponding values for the second compound shows that this substance, too, can hardly be described as liposoluble, because it dissolves in ether, benzene, and hexane to the extent of only 9 mg/l, 93 mg/l and 1 mg/l, respectively, and because, upon its partition between these solvents and water, only 0 2 to 0 6 % passes into the organic phase, i e more than 99 % remains in the aqueous phase. This compound is also rather sparingly soluble in water, the relevant figure being as low as 60 mg/l It therefore appears that this drug, like the first one, would be absorbed from the gastro intestinal tract only very poorly, if at all In fact, however, this is

Table 2

Solubility and partition

Solvents	H_2NCH-$CH(C_6H_4Cl)CH_2$ COOH		4,7 Phenanthroline-5 6-dione	
	Solubility mg/l	Partition % in organic phase	Solubility mg/l	Partition % in organic phase
Water	400	–	60	–
Butanol	10	40	160	61
Octanol	5	10	143	35
Methylene chloride	2	0.3	191	24
Ether	0 2	<0.3	9	0.6
Benzene	<0.2	<0.3	43	0 6
Hexane	0 0	<0 1	1	0.2

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Table 4

Partition of ferrioxamine

Aqueous phase	% in organic phase	
	Benzyl alcohol	Chloroform
Pure water	11 0	0 9
0.9 % NaCl solution	42.3	1 0
NaCl-saturated solution	95 4	4 1

tero-hepatic circulation does not provide an explanation for this lack of absorption

This example indicates either that notions concerning the unimpeded diffusion of liposoluble compounds through the intestinal barrier must be revised or, alternatively, that the solvents in question – such as butanol, octanol methylene chloride, and benzene – are not representative of the lipoid barrier in the intestine

Now for two examples illustrating how problematical the *in vitro* determination of water solubility and the partition of a substance between water and organic solvents may sometimes prove Presented in table 3 are data on the solubility of an antimalarial preparation It is a polycyclic compound which bears a basic sidechain and which is administered orally in the form of the hydrochloride Its solubility in a buffer solution amounts to 10 mg/l at pH 1 0 4 mg/l at pH 3, and 0 05 mg/l at pH 7 The most astonishing feature of this substance is the fact that in pure water it dissolves to the extent of 100 mg/l, whereas in physiological saline its solubility is only 0 1 mg/l In other words, the presence of 0 9 % sodium chloride in the physiological solution reduces its solubility by a factor of 1,000 In the case of this compound, it is impossible to decide which *in vitro* solubility from table 3 can be taken as representative for its solubility in the juices of the gastro-intestinal tract

Table 4 indicates the distribution of ferrioxamine (MEYER BRUNOT & KEBERLE 1968) between an aqueous phase and benzyl alcohol, as well as chloroform Pure water, physiological saline, and a saturated saline solution were used as the aqueous phase The effect exerted by the dissolved sodium chloride is quite striking. Whereas, upon partition between equal volumes of pure water and benzyl alcohol, only 11 % of the substance enters the benzyl alcohol phase and 89 % remains in the water, the *opposite is observed* when a saturated saline solution is employed 95 % enters the organic

not the case Both the first and the second substance are well absorbed by animals as well as man Astonishingly enough, despite its extremely low affinity for lipids, the first compound is absorbed not only in its entirety, but also at a relatively rapid rate

These two examples, to which many more could be added, reveal that liposolubility is by no means of such critical importance as a pre-condition for good enteral absorption What is more, good enteral absorption may also be possible where the drug's water solubility, too, is relatively low In the case of the two compounds to which reference has just been made, a liposolubility of only 0 2 mg/l of ether or benzene, and a water-solubility of less than 100 mg/l, is still sufficient to enable them to be well absorbed

The next example concerns a substance which appears to possess all the physico-chemical properties required to guarantee good absorption, but which nevertheless is not absorbed The substance in question is the ferrioxamine derivative P C F (*p*-chlorophenacetyl ferrioxamine) (MEYER-BRUNOT & KEBERLE 1968) This compound dissolves in water, butanol, octanol, and methylene chloride to the extent of more than 10,000 mg/l, the figure for its solubility in benzene being approximately 1,000 mg/l It is thus very readily soluble indeed, not only in water but also in the organic solvents mentioned In addition, it is neutral, i e it is not ionised In the lumen of the gut it is absolutely stable, and it is not metabolised when administered parenterally Its molecular weight is about 600 – which means that the molecule is not a particularly large one P C F, in fact, fulfils perfectly all the prerequisites for very good absorption Despite this, both in the rat as well as in the dog, oral doses are excreted in the faeces practically in their entirety Experiments on animals with a biliary fistula have shown that en-

Table 3

Water solubility of a polycyclic base
$(C_{21}H_{13}N_2S_2Cl_2)$ $NHC_2H_4N(CH_3)_2$ HCl

	mg per liter
Buffer pH 1	10
Buffer pH 3	0 4
Buffer pH 7	0 05
0 9 % NaCl solution	0 1
Pure water	100

Table 4

Partition of ferrioxamine

Aqueous phase	% in organic phase	
	Benzyl alcohol	Chloroform
Pure water	11 0	0 9
0 9 % NaCl solution	42 3	1 0
NaCl saturated solution	95 4	4 1

tero-hepatic circulation does not provide an explanation for this lack of absorption

This example indicates either that notions concerning the unimpeded diffusion of liposoluble compounds through the intestinal barrier must be revised or alternatively, that the solvents in question – such as butanol, octanol methylene chloride and benzene – are not representative of the lipoid barrier in the intestine

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phase, and only 5 % remains in the aqueous phase Here, once again, one is left wondering what partition coefficient should be taken as a measure of the lipophilic character of the compound

Having considered the question of lipid and water-solubility, let us now take a critical look at the third factor which is alleged to have a bearing on absorption, namely, the degree of ionisation of a compound It is generally supposed that in the case of ionisable compounds only the non ionic moieties of the dissociation equilibrium are capable of penetrating the epithelial membrane Hence the term "non-ionic diffusion". This idea is based, of course, on the assumption that only the molecules bearing no electrical charge are lipophilic, whereas the positively or negatively charged ions of organic acids or bases are not This assumption, however, does not always hold true Numerous organic acids and bases exist which are adequately liposoluble in ionic form The highly active analgesics of the benzimidazole series (HUNGER *et al* 1957) offer an example in point These compounds are strong bases and, as salts, they are also very readily soluble in organic solvents

There is yet another reason for doubting the general validity of the principle of non-ionic diffusion According to this concept, stronger bases with a pKa of 10 or above, which become completely dissociated in the gut, should prove to be very poorly absorbed, if at all This, however, is not borne out by the facts As a quanidine derivative, the antihypertensive compound guanethidine is a fairly strong base Although in the gut it remains in completely ionised form, it is nevertheless well absorbed by the enteral route (RAHN 1969)

A very important role in absorption is played by the fourth factor, to which insufficient attention is often paid, namely, the stability of a compound in the lumen of the gastro intestinal tract There are various reasons why a substance may be unstable here The reason is sometimes of a purely chemical nature, for example, the substance may become easily oxidised or it may be broken down by hydrolysis The latter applies, for instance, to some orally inactive penicillin and cephalosporin antibiotics (SULLIVAN & MCMAHON 1967), whose rapid breakdown by hydrolysis already occurs at pH 7

Another possibility is that the substance may prove unstable when acted upon by enzymes present in the stomach and intestine This seems to be the case, for example, with certain A C T H preparations which fail to act by the oral route When these same peptides are administered in the form of a nasal spray, on the other hand, they are absorbed (FELBER *et al* 1970)

The third principal possibility accounting for instability is that, following its ingestion, the substance may react with constituents present in the contents of the stomach and gut and in this way form sparingly soluble salts, complexes, or adsorbates

RIFAMPICIN

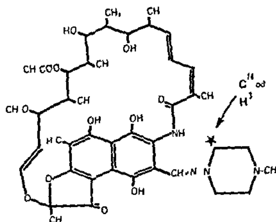


Fig. 1

The conclusion emerging from the foregoing observations is that very many factors, which it is difficult to assess and often impossible to correlate, are liable to affect the absorption of a substance If one wishes to know exactly how a new substance is absorbed, there is – for the moment, at least – virtually no alternative but to carry out experiments *in vivo*

At this point, an attempt will be made to show, by reference to a practical example how we study problems relating to absorption by the use of a method which has proved very reliable The example in question illustrates the effect which remnants of food stuffs in the stomach and gut exert on the absorption of a substance

The substance in this instance was rifampicin an orally active semi-synthetic antibiotic, and we wanted to find out first of all what percentage of the recommended therapeutic dose was in fact absorbed (RIESS *et al* 1969)

For this purpose as shown in fig. 1, we labelled the antibiotic with ^{14}C and tritium at the same site in the piperazine ring

A tests subject was given an oral dose of 300 mg of the ^{14}C labelled preparation on an empty stomach, plus at the same time an identical dose of the tritium labelled preparation, injected intravenously If the oral dose was absorbed quantitatively, then – according to Dost s law of corresponding areas – the surface area beneath the ^{14}C curve would have to be of the same size as that below the tritium curve In addition, the intravenous curve and the oral curve would have to intersect in the neighbourhood of their

maxima As revealed by the upper diagram in fig 2, the experiment in which the two doses were administered simultaneously showed that the postulates of Dost's law are perfectly fulfilled This, together with the fact that the maximum for the oral curve already occurs very early on, confirms with certainty that the orally administered dose of rifampicin must have been absorbed not only in its entirety, but also very rapidly In this experiment we also measured the drug's excretion in the urine In the lower diagram, the radioactivity excreted is indicated cumulatively as a percentage of the dose The fact that the patterns for both radioactivity curves are virtually identical affords further confirmation that the drug s absorption is quantitative

The same experiment was now repeated in a second test-subject This

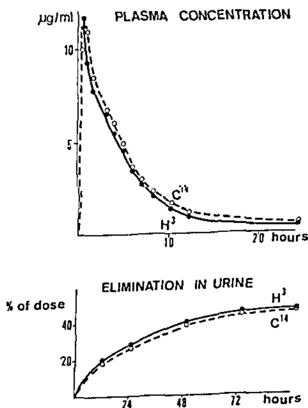


Fig 2

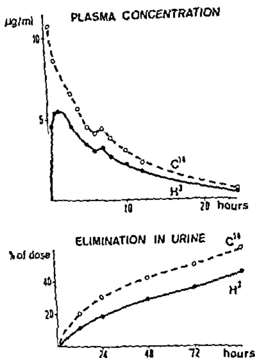
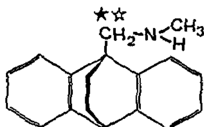


Fig 3

time, however, the oral dose was given, not on an empty stomach as in the first experiment, but shortly after a meal From the pattern of the blood concentration curves, plotted in fig 3, it can be seen at once that in this patient, absorption cannot have been quantitative The surface area under the oral curve is equivalent to only about 70 % of that under the intravenous curve That the dose was incompletely absorbed is also apparent from the urinary excretion Here, once again, the tritium curve reaches a heigh equivalent to about 70 % of that of the ^{14}C curve To calculate the portion of the dose absorbed from the gastro-intestinal tract, it is thus sufficient to determine the ratio of ^{14}C to ^{3}H radioactivity in the 48-hour urine a measurement which can be performed in a matter of minutes

BENZOCTAMINE



✱ C^{14} ☆ tritium

Fig 4

With this method, which we have named the "internal-reference method", we have also studied numerous other preparations and problems Here, however, only one further example will be described

This example is an interesting one, because it enabled us to confirm in man a rule concerning the absorbability of active substances from sup-

ELIMINATION OF BENZOCTAMINE IN URINE

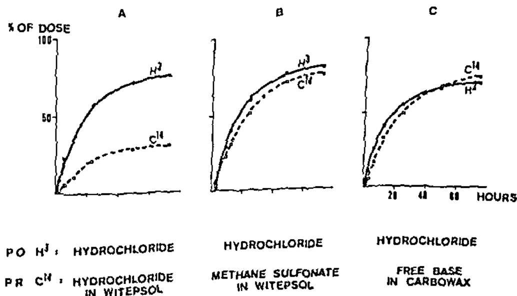


Fig 5



30'803 • 20 iv • 5 m



30'803 • 20 iv • 2 h

Fig. 6

positories a rule which we had previously deduced from an extensive series of studies carried out with sulphonamides

The rule runs as follows

If an active substance – whether an acid or a base – is liposoluble in non ionised form and water soluble in the form of a salt, it is the salt which will be better absorbed from fat soluble masses such as Witepsol, because it can be rapidly extracted from the Witepsol by the secretions in rectum Owing to its liposolubility, the non ionised form cannot be quickly extracted from the fatty mass instead, it is therefore more rapidly absorbed if it has been incorporated in a water soluble suppository mass such as Carbowax

Our new *tranquilliser, benzoctamine* was *available in the form of the* free base and the hydrochloride The free base is highly liposoluble whereas the hydrochloride – as was to be expected – is not although the hydrochloride crystals are admittedly rather poorly soluble *in water* We knew that both the free base as well as the hydrochloride are quantitatively absorbed when given by mouth (KEBERLE *et al* 1970) But we were



Fig 7

also looking for a suitable suppository formulation To compare various such formulations with respect to release of the active substance from them, benzoctamine was labelled with ^{14}C and ^{3}H at the same site, as shown in fig 4 We first tested a Witepsol suppository, in which the active substance was present in the hydrochloride form The test-subject was given simultaneously an oral dose of 10 mg ^{3}H-labelled benzoctamine in the form of the hydrochloride and a rectal dose of the same size consisting of ^{14}C-labelled benzoctamine hydrochloride incorporated in a Witepsol suppository

Shown in the first diagram in fig 5 is the radioactivity excreted in the urine It can be seen that much less of the rectally administered ^{14}C preparation is excreted than of the tritium-labelled preparation which was given by mouth We assumed that the poor rectal absorption of the active substance was probably due to the fact that the hydrochloride is only sparingly soluble in the watery secretions of the rectum If this assumption was correct, a readily soluble salt of benzocatamine, such as the methane



Fig 8

sulphonate for example, ought to be better absorbed in the rectum from a Witepsol suppository As revealed by the diagram in the middle, this is indeed the case The rectally administered ^3H labelled benzoctamine was in fact just as well absorbed as the oral dose of the ^{14}C preparation

We were now interested in discovering whether – in accordance with the rule – the other theoretically superior suppository formulation, *i e* the free base in Carbowax, would also yield a good result The third diagram shows that it did

Having cited this example, let us now abandon the problem of absorption and turn to the question of the distribution of a substance in the organism The numerous distribution studies which have thus far been carried out with substances of many different kinds have shown that certain connections exist between the physico-chemical properties of a compound and its distribution pattern It is known, for instance, that highly lipophilic substances such as anaesthetics, hypnotics, and sedatives become very rapidly distributed and that there are practically no barriers in the organism through which they cannot pass In organs which are rich in lipids and provided with a good blood supply, such as the brain, spinal *cord testes, and brown fat*, very high concentrations of these substances are built up within only a few minutes – concentrations which may be 10 to 50 times higher than the blood concentration If the lipophilic substance also displays basic properties, very high concentrations can in addi-

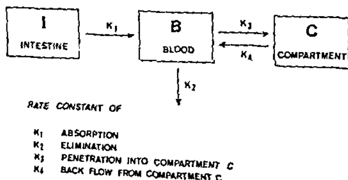


Fig. 9



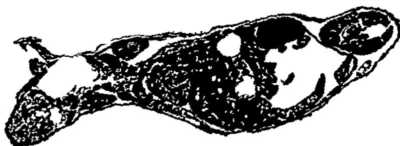
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Fig 8



UREA

Fig 11

Let us now take a look at a simplified pharmacokinetic model (fig 9) Compartment I represents the intestine, B the blood, and C a tissue compartment The so-called rate constants are indicated by the symbols k1 to k4 k1 indicates the speed at which the blood is invaded, k2 the elimination rate, k3 penetration into the tissue compartment, and k4 diffusion back from the tissue into the blood Let us now consider the blood and tissue concentration patterns of three hypothetical substances which differ only with respect to the constants k3 and k4 In fig 10 the uninterrupted curves represent the concentration in the blood, and the dotted curves the concentration in the tissue B1 and C1 belong to Substance I, B2 and C2 to Substance II, and so forth

In the case of Substance I, k3 and k4 are identical and, in absolute terms relatively small In other words, Substance I penetrates slowly into compartment C, and it diffuses back into the blood at the same slow rate This is what would happen, for example, with a non lipophilic substance which displays no particular affinity for the brain and therefore passes slowly through the blood brain barrier and diffuses out of the brain again at an equally slow rate In the case of such substances, the concentration in the blood at the point in time indicated by t1 is far higher than in the compartment, at t2 both concentrations are the same, it is remarkable, however, that at t3 the concentration in the compartment – i e the concentration in the brain in this particular example – is higher than in the blood

The autoradiograms reproduced here, which were obtained at various intervals following the injection of radioactive urea, illustrate a case of this kind

In the first autoradiogram (fig. 11), which was taken 5 minutes after the injection, the concentration of ^{14}C-labelled urea in the blood is – as at the time t1 – much higher than in the brain compartment The upper

tion be found in the lungs, kidneys, and adrenals, as illustrated by the autoradiogram shown in fig 6, which was obtained with benzoctamine

Lipophilic substances which – like the hypolipidaemic agent CIBA-13,437-Ba (HESS & BENZCE 1968) – are at the same time also weak acids, attain high concentrations in the liver and show an affinity for the plasma albumin (fig 7)

Substances, such as rifampicin, which are excreted in the bile or which are rapidly metabolised in the liver also attain high concentrations in the liver Strongly basic, hydrophilic compounds of the bis-guanidine type are capable of reaching in the liver, kidneys, and adrenals concentrations that are several thousand times higher than in the blood (fig 8)

This poses the question as to what processes are responsible for the fact that the concentrations of substance built up in one particular kind of tissue may be higher than in the blood The distribution of a compound in the organism generally takes place by simple diffusion in accordance with first-order kinetics

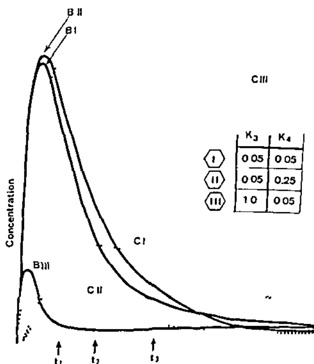


Fig 10

example in point, one could take the autoradiograms obtained with benzoctamine (fig 6) Here, the relatively higher concentration in the brain persists throughout the entire time of observation

By way of conclusion, mention should be made of a few other processes which may lead to the build up of a substance in the tissues Four such processes are outlined schematically in fig. 13 The first applies to Substance A, which is lipophilic and penetrates easily through a lipid barrier into the compartment. Substance A, however, is unstable and is converted within the compartment into a metabolite M, which cannot penetrate the barrier so easily as the parent compound A and therefore accumulates inside the compartment This, as we have shown, is the case with thalidomide, for instance (KEBERLE *et al* 1965) Thalidomide is capable of penetrating into the blastocyst, where it then becomes hydrolysed Since the resultant polar metabolites cannot pass out again so easily through the membrane of the blastocyst, *they proceed to accumulate in the blastocyst*

In the second process, Substance B penetrates into the compartment, where it becomes transformed and polymerised The high molecular polymerisate cannot escape from the compartment and therefore accumulates

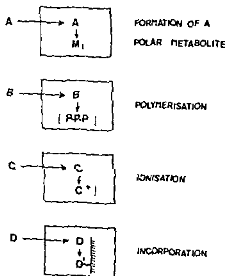


Fig. 13

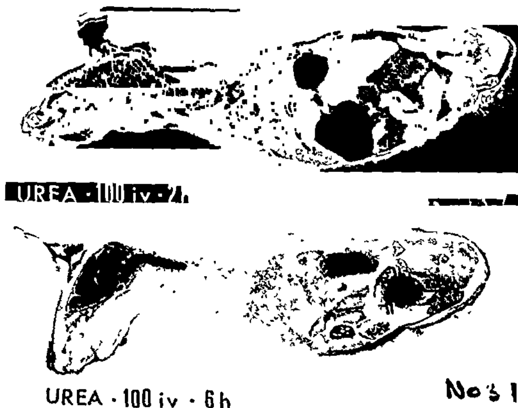


Fig 12

picture in fig. 12 shows that, as at time t2 *in the diagram*, the concentrations in the brain and blood after 2 *hours are identical*, whereas the lower picture reveals that 4 hours later, as *at time t3*, the concentration in the brain is higher than in the blood. If, as *often happens* in published papers, one had been shown only the *last autoradiogram*, one might well have been misled into supposing that urea is a *substance* displaying a strong affinity for the central nervous system – *which*, of course, is certainly not so.

In the case of Substance II, k4 is *greater than* 43 in the diagram (*fig. 10*). This, *for example*, *would* be a substance showing very little affinity for the tissue in the compartment, as a *hydrophilic* substance with respect to the brain. As revealed by the patterns of the curves designated *BII* and *CII*, the blood concentration of such a substance is always higher than the concentration in the compartment

With Substance *III*, k3 is greater than k2 Here, the substance has an affinity for the tissue in the compartment – an affinity such as a highly lipophilic compound displays for the brain. The concentra-
t1, t2 and t3 are far higher in the compartment than in the

REFERENCES

Meyer Brunot, H G & H Keberle Biliary excretion of ferrioxamines of varying liposolubility in perfused rat liver *Amer J Physiol.* 1968, 214, 1193–1200

Hunger A J Keberle A. Rossi & K. Hoffmann Synthese basisch substituierter anal getisch wirksamer Benzimidazol Derivate *Experientia* 1957 13 400

Rahn K. G Untersuchungen uber die Ausscheidung von Metaboliten des Guanethidin (Ismelin) im Harn von Hypertonikern *Verh dtsch Ges inn Med* 1969 75 157 159

Sullivan H R & E. R McMahon Metabolism of oral cephalotin and related cephalosporins in the rat *Biochem J* 1967, 102 976

Felber J P M L. Aubert & R. Degaudlaume Administration by nasal spray of an 18 amino acid synthetic polypeptide with corticotropic action. *Experientia* 1970 25 1195–1196

Riess W., K. Schmid, H. Keberle L. Dettli & P Spring Pharmacokinetic studies in the field of rifamycins. *Proc of the sixth Int Congress of Chemotherapy Tokyo,* August 10–15 1969

Keberle H W Riess, K. Schmid P Imhof & T G Rajagopalan Metabolismus und Pharmakokinetik des Tacitin *Entspannung – neue therapeutische Aspekte* Ein internationale Symposium St Moritz, 16 Januar 1970 herausgegeben von der CIBA Aktiengesellschaft.

Hess, R. & L. W Benzce Hypolipidaemic properties of a new tetralin derivative (CIBA 13 437 Su) *Experientia* 1968, 24, 418–419

Keberle H J W Faigle H Fritz, F Knusel, P Loustalot & K. Schmid Theories on the mechanism of action of thalidomide *Biolog cal Council Symposium on embriopathic activity of drugs* 1965 p 210 Ed. J M Robson F Sullivan and R. L Smith J & A. Churchill Ltd., London.

Faigle, J W & H. Keberle The metabolic fate of CIBA 32,644-Ba. *Acta Tropica* Separatum Supplementum 1966, 9, 8–22

within it This is the way in which the schistosomicide niridazole (FAIGLE & KEBERLE 1966) behaves in the parasite or in the eggs of the parasite

The third process is exemplified by Substance C, which is a lipophilic but ionisable compound, e g a weak organic base The non ionised molecules penetrate into the compartment, where they then encounter an acid pH or acid groups Substance C thus becomes ionised or bound in the form of a salt to the acid groups, with the result that it is retained inside the com partment in a higher concentration (KEBERLE *et al* 1966) This is probably what happens in the case of highly lipophilic bases which build up high concentrations in the lungs

In the fourth process, Substance D becomes chemically bound to, or incorporated in, a particular structure The retention and accumulation of certain bisguanidines in the liver, thymus, and adrenals is probably at tributable to this type of mechanism

Summary

The importance of the following physico chemical factors is critically discussed lipid and water solubility, degree of ionisation, chemical stability, and molecular weight It is pointed out that descriptions of these factors couched in general terms do not prove of much use in practice, i e in predicting the behaviour of a drug in the organism, and that for this purpose one first has to define more closely the scope and the contribution of each individual factor To illustrate the difficulties encountered in this connection, examples are cited which appear at first sight to contradict the generally accepted rules

A new and very reliable method for the study of problems relating to absorption is presented and illustrated with two practical examples

Numerous distribution studies have shown that connections exist be tween the physico chemical properties of a compound and its distribution pattern The distribution pattern of neutral, basic, and acidic lipophilic substances, as well as of strongly basic hydrophilic compounds is de scribed in the light of practical examples

The question as to what processes are responsible for the fact that the concentrations of a substance built up in one particular kind of tissue may be higher than in the blood is discussed by reference to a simplified pharmacokinetic model Finally, different mechanisms which may lead to the build up of a substance or its metabolites in a tissue compartment are outlined schematically

to albumin A general rule, however, seems to be that positively charged drugs have low binding parameters (defined below) Hardly any such drug has been studied which binds to serum albumin to a degree comparable to anionic drugs Therefore, when studying physico-chemical factors influencing drug protein binding we have chosen to work with sulfa drugs which are known to bind effectively

Material and Methods

All sulfa drugs studied in this work were of the quality on the market used without further purification Human serum albumin was of the quality purchased from *AB* Kabi Stockholm Sweden. Other chemicals were of analytical grade

Degree of binding was determined by equilibrium dialysis using a plexi glass cell with two compartments and a cellophane membrane (Union Carbide Corp) Each compartment had a depth of 4 mm and a diameter of 50 mm, which contained 6 00 ml of solution thus leaving a small volume of air During equilibration (15 hrs were used although 5 hrs would have been sufficient) the cells were rocked gently in a thermostated water bath All experiments were made at an ionic strength 0 2 and 25 00 ± 0 05° Phosphate buffers were used in the pH range 5.5 – 8 0 At higher pH borate buffers were used In all dialysis experiments the concentration of albumin was 1 0 × 10^{-4} M, based on a molecular weight of 69 000 The same batch of albumin was used in all experiments. The initial sulfa drug concentration ranged from 1 00 × 10^{-5} M to 1 00 × 10^{-3} M

All pKa values were determined at the same ionic strength and temperature as used in the dialysis experiments by means of a spectrophotometric technique proposed by BATES & SCHWARZENBACH (1954)

The equilibrium concentrations of sulfa drug in the dialysis runs were also determined spectrophotometrically after diazotizing and coupling with N(1 naphtyl) ethylene diamine according to BRATTON & MARSHALL (1939)

Results and Discussion

Thirteen sulfa drugs have been investigated which are more or less related to each other The usual way to present the data is to plot them according to SCATCHARD (1949) One example from the present work is shown in fig 1 Here $\bar{n}$/A is plotted versus $\bar{n}$, where $\bar{n}$ is the mean number of small molecules bound per molecule of protein A is the concentration of unbound drug at equilibrium A straight line appears when only one type of binding sites is available If one gets a curved line this indicates the presence of two or more types of binding sites

The degree of binding at varying pH is best presented in a pH profile as in fig. 2 where degree of binding, using a suitable parameter, is plotted versus pH This figure shows that there is a strong increase in binding when pH is increased from the acidic to the basic side of pKa value of the

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Some Physico-Chemical Factors Influencing the Binding of Sulfonamides to Human Albumin *in vitro*

By

Allan Ågren, Rune Elofsson and Sten Ove Nilsson

Abstract Thirteen sulfa drugs have been investigated Their degree of binding to human serum albumin has been studied *in vitro* at varying pH It was found that the degree of binding increased remarkably near the pKa value A drug with a low pKa has a comparatively high degree of binding at physiological pH whereas a drug with a high pKa has a low degree of binding at this pH It is shown that a good fit of experiments at a number of pH values is obtained when a modified Scatchard equation is used

$$\bar{n} = \frac{N_1 k_1 A_{on}}{1 + k_1 A_{on}} + \frac{N_2 k_2 A_o}{1 + k_2 A_o}$$

This shows that the uncharged species is hardly bound at all The variation in binding ability among similar ions also depends on the hydrophobic bonds which are correlated to the partition coefficient of the drug between octanol and water A quantum mechanical approach has started which shows a correlation between degree of binding and distribution of electronic charge within the drug molecules Some data are presented

Key words Sulfa drugs – albumin – protein binding

The binding of drugs to plasma proteins plays an important role in pharmacokinetics A great number of publications have appeared in this field (KLOTZ & WALKER 1948, MEYER & GUTTMAN 1968 SCHOLTAN 1964 SEYDEL 1966)

There is, however, still a need for more exact investigations in order to be able to predict the degree of binding of new drugs

In a previous work (AGREN *et al* 1970) the influence of Donnan effect and ionic strength has been investigated

Most drugs are either weak bases or weak acids which means that at physiological pH they are partly ionized Since serum albumin contains a great number of negatively or positively charged groups one would expect that all drugs being positive or negative ions should bind rather strongly

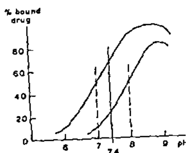


Fig 3 Theoretical curves of expected pH profiles for drugs with pKa = 7 0 or 8 0 respectively

When Scatchard plots are drawn at different pH values for one drug a number of curves are obtained as shown in fig 1 and 4 This was thought to be due to different binding abilities for ionized and non-ionized drug. It turned out, however, to be impossible to find parameters satisfying all curves on this assumption If on the other hand one assumes that only the ionized drug is bound, a modified equation should be valid

$$\bar{n} = \frac{N_1 k_1 A_{on}}{1 + k_1 A_{on}} + \frac{N_2 k_2 A_{on}}{1 + k_2 A_{ion}}$$

If $\bar{n}/A_{on}$ is plotted versus $\bar{n}$ one should get one curve for all data at all pH values Fig 5 shows the data of sulfamerazine and fig 6 of sulfametine (sulfamethizolum NFN) By using a digital computer program the values of the four parameters N_1, N_2, k_1 and k_2 were determined which give the best fit to the data points (least squares) Relative errors are of the order

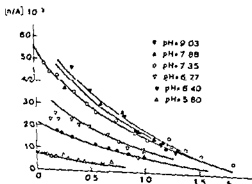


Fig. 4 Scatchard plots at varying pH values for sulfamerazine Concentrations in M Albumin concentration = 1 0 10 4

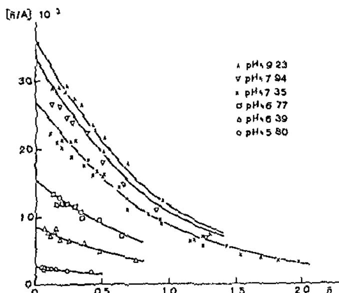


Fig 1 Scatchard plot of dialysis experiments with sulfametine Concentrations in M Albumin concentration = 1 0 10⁻⁴

drug This qualitatively shows that the anionic form is more bound than the uncharged species At pH greater than 9 the binding is less again due to changes in the protein

Fig 3 shows that at pH ≈ 7 4 the degree of binding depends on the pKa of drug If pKa is less than 7 the drug is relatively extensively bound If pKa is higher than 8 it is less bound This has been shown earlier (SCHOLTAN 1964)

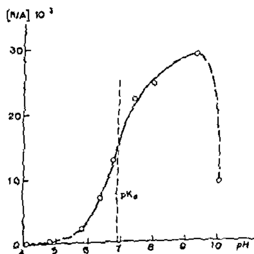


Fig 2 pH profile of sulfamerazine Albumin concentrat = 1 0 10⁻⁴ M n = 0 20

Table I

Binding parameters ($\Sigma N_i k_i$) and charge distribution of some *sulfa drugs*.

$H_2N-C_6H_4-SO_2$ NH R

No	R	10^{-4} Σ N k	Charge of term nal mo n atom
1	N, N	0 5	0 22
2	N, N, CH_3	4 0	-0 37
3	N, N, CH_3	3 5	-0 30
4	N, N, CH_3, CH_3	5 6	0 39•2
5	N, N, OCH_3	6 7	0 80
6	N, N, CH_3, CH_3	15	
7	N, N, OCH_3, OCH_3	34	
8	N, N, CH_3O	1 5	

Recently SWANEY & KLOTZ (1970) showed that serum albumin contains a peptide sequence forming a cavity with one tryptophan residue in the bottom and positive charges at the border of the cavity Negative drug molecules will fit into the cavity Human serum albumin contains only one tryptophan unit which fits well with the observation that many drugs have only one binding site in albumin One may assume that tryptophan forms hydrophobic bonds to the drug Hydrophobic bonds may be an overlapping of pi-electron clouds from the two molecules or an entropy effect When the hydrophobic bond is formed a number of water molecules are set free from the hydrated molecules resulting in an increased entropy value of the system

It has also been suggested that there should be a close relationship between the tendency of forming hydrophobic bonds and the partition coefficient of the drug between an organic solvent and water We have made such comparisons and some data will be published elsewhere (AGREN et al 1971) No data will be presented in the present paper but we found that such a correlation does exist as long as homologues are studied

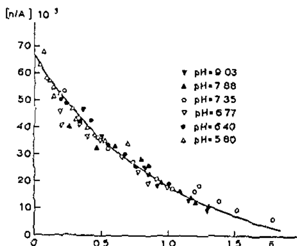


Fig 5 Sulfamerazine plot using the equation presented in text Same data as in fig 4 Solid line calculated using following parameter values $N_1 = 0.7$, $N_2 = 2.2$, $k_1 = 4.2 \cdot 10^4 M^{-1}$, $k_2 = 3.0 \cdot 10^3 M^{-1}$

of 5 % The solid lines of fig 5 and 6 have been calculated using the parameter values given in the figures, valid for all pH values Similar well fitting curves have also been found for other sulfa drugs studied Another way of presenting the use of this modified equation is shown in fig 1 and 4 where the solid lines have been calculated from the same parameters as given in fig 6 and 5, respectively

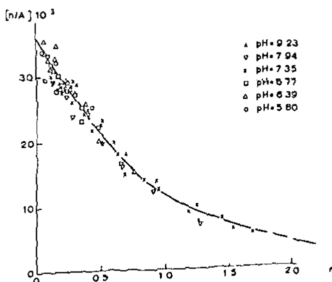


Fig 6 Sulfametine plot using the equation presented in text Same data as in fig 1 Solid line calculated using following parameter values $N_1 = 0.46$ $N_2 = 1.54$, $k_1 = 1.0 \cdot 10^5 M^{-1}$, $k_2 = 1.2 \cdot 10^4 M^{-1}$

Table I.

Binding parameters ($\Sigma N_i k_i$) and charge distribution of some sulfa drugs.

H_2N-⟨benzene⟩-SO_2 NH R

No	R	$10^{4}\Sigma N_i k_i$	Charge of terminal mean atom
1	pyrimidinyl	0 5	-0 22
2	CH_3-pyrimidinyl	4 0	-0 37
3	CH_3-pyrimidinyl	3 5	-0 30
4	CH_3, CH_3-pyrimidinyl	5 8	-0 39·2
5	OCH_3-pyrimidinyl	6 7	-0 80
6	CH_3, CH_3-pyrimidinyl	19	
7	OCH_3-pyrimidinyl	34	
8	OCH_3, CH_3O-pyrimidinyl	1 5	

Recently SWANEY & KLOTZ (1970) showed that serum albumin contains a peptide sequence forming a cavity with one tryptophan residue in the bottom and positive charges at the border of the cavity Negative drug molecules will fit into the cavity Human serum albumin contains only one tryptophan unit which fits well with the observation that many drugs have only one binding site in albumin One may assume that tryptophan forms hydrophobic bonds to the drug Hydrophobic bonds may be an overlapping of pi-electron clouds from the two molecules or an entropy effect When the hydrophobic bond is formed a number of water molecules are set free from the hydrated molecules resulting in an increased entropy value of the system

It has also been suggested that there should be a close relationship between the tendency of forming hydrophobic bonds and the partition coefficient of the drug between an organic solvent and water. We have made such comparisons and some data will be published elsewhere (AGREN et al 1971) No data will be presented in the present paper but we found that such a correlation does exist as long as homologues are studied.

In table 1 some data in this investigation are surveyed It may be seen that one or two methyl groups increase the binding tendency One or two methoxy groups have a stronger effect than the methyl groups Methoxy in *ortho* position (drug no 8) decreases the binding capacity probably due to a steric hindrance of the acidic group $-SO_2$ NH– When one ring nitrogen is moved from position 2 to position 4 from the amido nitrogen a strong increase in binding capacity is observed although hardly any change in pKa value is observed In table 1 the binding ability is expressed as ΣN k This parameter is the intercept of the Scatchard plot and represents the formation constant of a complex between drug and protein at zero drug concentration

From a practical point of view the interesting thing to know is the percentage of unbound drug in plasma Table 2 shows some data calculated from the pKa values and binding parameters ($\Sigma N_i k_i$) found in the present work The numbers are valid in a 5 % albumin solution at pH = 7 4 and 25° and at very low drug concentrations The estimated errors given in the table have been calculated assuming a mean error of 5 % in the value $\Sigma N k$ (for sulfadimethoxine 20 %) and 0 02 pH units error in pKa It may be seen that most of the drugs are extensively bound

For sulfanilamide the numbers of table 2 are rather crude estimations but nevertheless the data are interesting since they differ remarkably from

Table 2

Calculated data of sulfa drugs in 5 % albumin at pH = 7 4 and 25° from binding parameters and pKa values found in this work The numbers are limiting values at zero concentration

Name	% Bound drug	% Free base	% Free acid
Sulfanilamide	(5)	0	95
Sulfadiazine	76 4 ± 1 2	21 7 ± 1	1 9 ± 0 2
Sulfaperine	95 0 ± 0.3	4 2 ± 0 2	0 76 ± 0 08
Sulfamerazine	94 9 ± 0 3	3 9 ± 0 2	1 2 ± 0 1
Sulfadimidine	94 1 ± 0.5	2 3 ± 0 1	3 6 ± 0 4
Sulfametine	97 5 ± 0 14	2 1 ± 0 1	0 38 ± 0 04
Sulfisomidine	97 8 ± 0 15	0 9 ± 0 05	1 3 ± 0 1
Sulfadimethoxine	99 7 ± 0 1	0 3 ± 0 07	0 010 ± 0 003
Sulfalene	90 8 ± 0 5	8 7 ± 0 4	0 54 ± 0 05
Sulfamethoxypyridazine	98 1 ± 0 1	1 2 ± 0 05	0 72 ± 0 07
Sulfamethizole	97 6 ± 0 1	2.4 ± 0 1	0 018 ± 0 002
Sulfafurazole	98 2 ± 0 1	1 8 ± 0 1	0 006 ± 0 001
Carbutamide	96 7 ± 0 2	3 2 ± 0 2	0 072 ± 0 007

all the others This is due to its high pKa value (10 65) and thus it is not ionized at all at this pH That part of the unbound drug which is non-ionized may pass through lipid membranes and thus the numbers in the last column of table 2 will determine the *rate* of distribution to the rest of the body

Sulfadimidine and sulfametine for example are both bound to almost the same degree (94 and 98 % respectively) However, the percentage of unbound acid is very different (3 6 and 0 38 % respectively)

An attempt has also been made to correlate the binding ability with the electron distribution within the sulfa drugs A quantum mechanical investigation has started We use an extended Huckel approximation method and a computer program written by Dr R. Manne in Uppsala (unpublished) The negatively charged groups bound to the sulfur atom of the sulfonamide derivative have been used in the calculations Since the sulfanilic group is identically the same in all compounds studied the calculated numbers should be suitable for comparisons but are not true values for the intact molecules Only a very small number of drugs have been studied until now but the data seem to show a good correlation with the binding parameters This work will continue

Acknowledgements

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Sulfadiazine, sulfamerazine, sulfadimidine and carbutamide by Vitrum AB, Stockholm Sweden Sulfaperine by Merck Darmstadt, W Germany Sulfametine by Bayer, Leverkusen, W Germany Sulfisomidine (sulfoisodimidinum NFN) by Ciba, Basle, Switzerland Sulfadimethoxine and sulfafurazole by Roche Basle Switzerland Sulfalene by Kabi, Stockholm, Sweden Sulfamethoxypyridazine by Astra, Sodertalje, Sweden Sulfamethizole by Pharmacia, Uppsala, Sweden

REFERENCES

Bates R G & G Schwarzenbach. Die Bestimmung thermodynamischer Aciditatskonstanten *Helv Chim Acta* 1954 37 1069–1079

Bratton A. C. & E. K. Marshall, jr A new coupling component for sulfanilamide determination *J Biol Chem* 1939 128, 537–550.

Klotz, I M & F M. Walker The binding of some sulfonamides by bovine serum albumin *J Amer Chem Soc* 1948 70, 943–945

Meyer M C & D E. Guttman The binding of drugs by plasma proteins. *J Pharm Sci* 1968, 57 895–918.

Scatchard, G The attraction of proteins for small molecules and ions. *Ann N Y Acad Sci* 1949, 51, 660–672.

Scholtan W Die Bindung der Sulfonamide an Eiweisskorper 3 Mitteilung Abhangigkeit der Eiweissbindung vom pH der Losung von der Konstitution der Sulfonamide und von der Art des Eiweisskorpers (1 Teil) *Arzneimittel Forsch* 1964 14 348–356

Scholtan, W Die Bindung der Sulfonamide an Eiweisskorper 1 Mitteilung Abhangigkeit der Eiweissbindung vom pH der Losung, von der Konstitution der Sulfonamide und von der Art des Eiweisskorpers (2 Teil) *Arzneimittel Forsch* 1964 14, 469–473

Seydel J Molekulare Grundlagen der Sulfonamidwirkung *Arzneimittel Forsch* 1966 16 1447–1453

Swaney, J B & I M Klotz Amino acid sequence adjoining the lone tryptophan of human serum albumin A binding site of the protein *Biochem* 1970, 9, 2570–2574

Ågren, A, R Elofsson, U Meresaar & S O Nilsson Complex formation between macromolecules and drugs III A study of the influence of ionic strength and pH Binding between Aptin®, nortriptyline, sulfamethizole or triplenamine and serum albumin, poly arginine, poly lysine, poly ornithine, protamine or dextran sulfate *Acta Pharm Suec* 1970, 7, 105–112

Ågren A, R Elofsson & S O Nilsson Correlation of binding parameters for the binding of sulfa drugs to human serum albumin (HSA) with partition coefficients thermodynamic values and quantum mechanical data *Acta Pharm Suec* 1971 in press

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Effect of Complex Formation on Drug Absorption

By

Gerhard Levy

Many organic drug molecules can form relatively weak reversible associations with one or more molecules of another drug, surfactant, protein, or other species (MARTIN *et al* 1969) The drug complexes thus formed can differ appreciably from the free drug with respect to such physical-chemical characteristics as solubility, diffusivity, size, electrical charge, and lipoid water partition coefficient Such differences imply corresponding differences in the absorbability of the free drug and drug complex from the gastrointestinal tract Unintentional and unanticipated interactions of drugs with other constituents of their dosage form, with ingested food or other normal constituents of the gastrointestinal tract, or with other drugs may lead to the formation of poorly absorbed complexes and consequent drug inactivation The poor absorption of tetracyclines in the presence of calcium (KUNIN & FINLAND 1961) and the ineffectiveness of a commercial orally administered iron-carbohydrate complex (DIAMOND *et al* 1963) are examples of such adverse interactions On the other hand, there is the exciting possibility that certain relatively lipoid-insoluble drugs which are poorly absorbed from the gastrointestinal tract may be rendered more absorbable by formation of relatively lipoid soluble complexes The complexing agent would thus serve as a carrier, analogous in some respects to the real or hypothetical carrier in physiologic transport processes Explorations of this possibility are still in their beginning, and the feasibility of this technique in medicine is only speculative at present Based on studies in the author s laboratory over a number of years (LEVY & REUNING 1964, LEVY & MATSUZAWA 1965, LEVY *et al* 1966, REUNING & LEVY 1967, LEVY & ANELLO 1968, LEVY & MROSZCZAK 1968, REUNING & LEVY 1968a & b, REUNING & LEVY 1969, ANELLO & LEVY 1969, HAYTON *et al* 1970), three different types of drug complexes will be described here the salicylamide caffeine complex which is less lipoid soluble and more slowly absorbed than salicylamide itself (REUNING & LEVY 1968b & 1969) the secobarbital (meballymalum NFN) -polysorbate 80 and 4-aminoantipyrine polysorbate 80 complexes which are not at all absorbed due to the size and lipoid insolubility

of the surfactant micelle (LEVY *et al* 1966, ANELLO & LEVY 1969), and the complex of prednisone with certain N,N dialkylpropionamides which is more rapidly absorbed than the free steroid (HAYTON *et al* 1970)

The salicylamide caffeine complex

Salicylamide and caffeine form a complex in aqueous solution with a stability constant of 41 L/M at pH 5 and 37°, based on a 1 1 stoichiometry (REUNING & LEVY 1968c) The apparent lipoid water partition coefficient of this complex is much smaller than that of salicylamide and similar to that of caffeine (REUNING & LEVY 1968b) Fig 1 and 2 show the cumulative transfer of the two drugs, alone and in the presence of one another across the everted small intestine of the rat

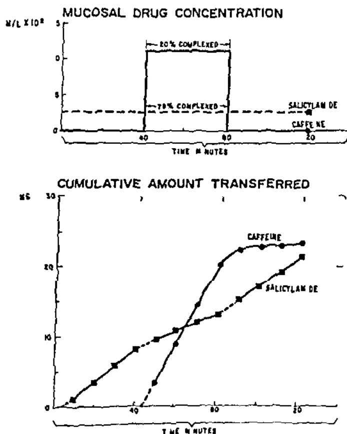


Fig 1 Effect of complex formation with caffeine on the transfer of salicylamide across the cannulated everted intestine of the rat In the upper portion of the figure the concentrations of salicylamide and caffeine in the mucosal solution are plotted as a function of time In the lower portion of the figure the cumulative amounts of salicylamide and caffeine transferred across the intestine are plotted as a function of time (From REUNING & LEVY 1968b)

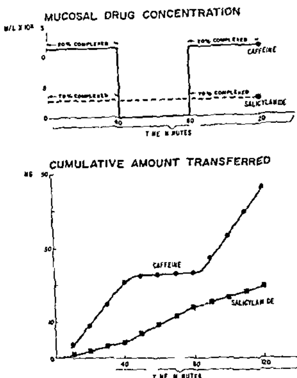


Fig 2. Effect of complex formation with caffeine on the transfer of salicylamide across the cannulated everted intestine of the rat. Details as in fig. 1 except that the order of addition of the complexing agent was changed (From REUNING & LEVY 1968b)

The transfer rate of salicylamide was decreased by caffeine while the transfer rate of the latter was not affected by salicylamide The over all rate constant for salicylamide absorption (k_{app}) is a function of the rate constants for absorption of free drug (k_{sam}) and drug complex ($k_{complex}$) and the fraction of salicylamide which is free (F_f) and complexed (F_c)

$$k_{app} = k_{sam}(F_f) + k_{complex}(F_c) \quad (1)$$

Rearrangement of this equation and division of each side by k_{sam} yields

$$\frac{k_{app}}{k_{sam}} = \frac{k_{complex}}{k_{sam}} + F_f\left[1 - \frac{k_{complex}}{k_{sam}}\right] \quad (2)$$

Therefore, a plot of k_{app}/k_{sam} vs F_f should be linear, permitting a determination of $k_{complex}$ This is indeed the case, as shown in fig. 3, which is based

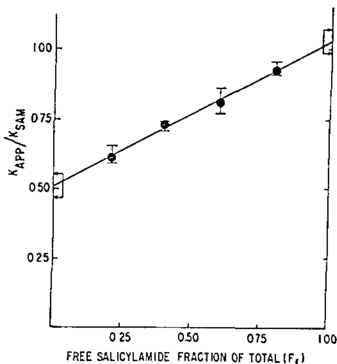


Fig 3 Least squares plot of k_{app}/k_{sam} as a function of F_f. Vertical bars indicate the range of the experimental values (4 experiments per data point) and arrows show the 95 % confidence intervals of the two intercepts, as calculated from the 16 individual experiments (From REUNING & LEVY 1969)

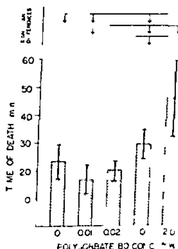


Fig 4 Effect of polysorbate 80 on the time of death of goldfish immersed in 0.020 % sodium secobarbital solution (pH 5.9, 20°) Mean of 10 fish each. Vertical bars indicate ± 1 standard deviation. Arrows connect values which differ significantly ($P < 0.05$) from one another (From LEVY *et al* 1966)

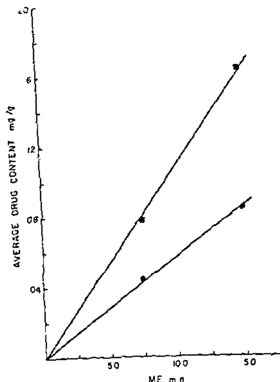


Fig 5 Effect of polysorbate 80 on the absorption of 4-aminoantipyrine by goldfish. Fish were immersed in 250 mg % 4-aminoantipyrine with (squares) or without (circles) 0 01 % polysorbate 80 in 0 05 M Tris buffer pH 7 0 at 20° ± 0 5° Each point represents the average of five fish. (From ANELLO & LEVY 1969)

on a series of experiments at different caffeine but constant salicylamide concentrations These data show that the salicylamide-caffeine complex is transferred as such across the intestinal wall, but half as rapidly as salicylamide and at a similar rate as caffeine

Micellar complexes of secobarbital or 4 aminoantipyrine with Polysorbate 80

An interesting pharmacologic technique with goldfish (LEVY & GUCINSKI 1964) was used to study the effect of polysorbate 80 on drug absorption As shown in fig 4, low concentrations of the surfactant enhance and high concentrations retard the absorption of secobarbital, as evidenced by the time of death of the fish when immersed in secobarbital solutions containing

various concentrations of polysorbate 80 Low concentrations of the surfactant enhance the absorption of the barbiturate by increasing the permeability of the biologic membranes At high concentrations of polysorbate 80, micellar complexation reduces the thermodynamic activity of the drug and thereby decreases its rate of absorption The over all effect depends on the relative contribution of these two opposing effects It can be shown that correction of the apparent absorption rate constant for the degree of micellar binding at high polysorbate concentrations yields essentially the same value as obtained at low surfactant concentrations when only the membrane permeability enhancing effect is evident Thus, the drug micelle complex is not absorbed as such

Studies with 4-aminoantipyrine showed by direct chemical assay that low concentrations of polysorbate 80 enhanced the absorption as well as the

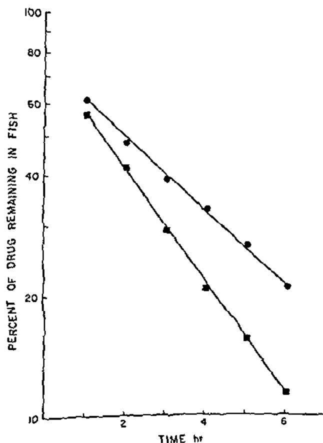


Fig 6 Effect of polysorbate 80 on exsorption of 4 aminoantipyrine from goldfish after absorption of approximately 1 mg/g body weight by immersion in 4-aminoantipyrine solution Circles represent exsorption into 0 05 M Tris buffer pH 7 0 (average of six fish), squares indicate exsorption into buffer containing 0 01 % polysorbate 80 (average of seven fish) All experiments at 20° ± 0 5° (From ANELLO & LEVY 1969)

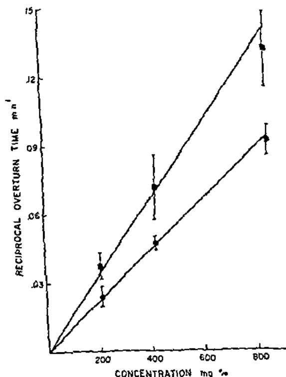


Fig. 7 Effect of polysorbate 80 on reciprocal overturn time in goldfish Squares, 4-aminoantipyrine with 0 01 % polysorbate 80, circles, without polysorbate 80 All solutions at pH 7 0 Each point is the average of five fish vertical bars represent ±1 S D (From ANELLO & LEVY 1969)

exsorption rate of 4-amino-antipyrine in goldfish (fig 5 and 6) Absorption studies based on a pharmacologic endpoint (fig 7) yielded similar results (table 1) These results are interesting from a theoretical point of view and illustrate an important principle, but they should not be extrapolated in the quantitative sense to other species Subsequent studies have shown that low concentrations of polysorbate 80 do not enhance drug absorption in rats (LEVY & PERALĂ 1971) although higher concentrations retard absorption in rats as in goldfish, i e , by micellar complexation (LEVY & REUNING 1964)

Prednisone N,N dialkylpropionamide complexes

Prednisone and prednisolone form complexes with N,N-dialkylpropion amides of which all but the methyl derivative are more lipoid soluble than

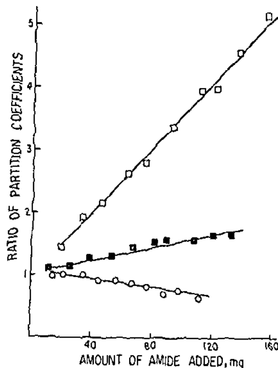


Fig 8 Apparent partition coefficient of prednisone at 25° as a function of the amount of amide added to a system of 2 ml isopropyl myristate and 2 ml water ○ N,N dimethylpropionamide, ■, N,N diethylpropionamide, □, N,N di n propylpropionamide

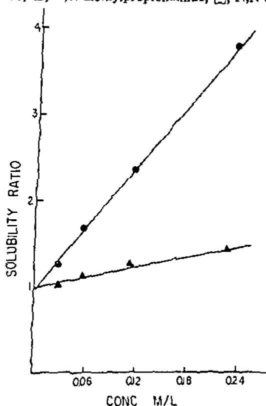


Fig 9 Apparent solubility of prednisone at 25° as a function of N,N di n propyl propionamide concentration in water (▲) and isopropyl myristate (●)

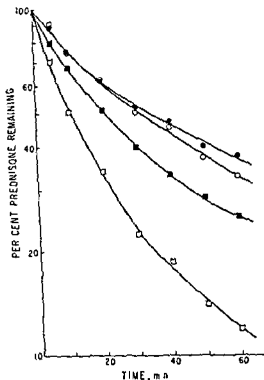


Fig 10 Effect of various amides (initial concentration 2.8×10^{-2}M) on the absorption of prednisone (initial concentration, 5×10^{-4}M) from the rat intestine ●, Control, ○ Methyl ■ Ethyl □ n Propyl as the substituent on N,N-dialkylpropionamide

the steroids themselves and are much stronger in lipoid solvents than in water (fig 8 and 9) These complexes are absorbed considerably more rapidly than the free steroid from the *in situ* rat intestine (for example, fig 10) This is not due to a direct effect of the amide on the barrier properties of the intestine, since the amides have no effect on the absorption of drugs such as caffeine with which do not interact

These are examples of a type of drug interaction which is seldom recognized Its present implication is primarily adverse interference with the absorption and activity of certain drugs due to the formation of poorly absorbable complexes. In the future there is the possibility of using complexation for the controlled retardation of absorption, perhaps to reduce local or systemic adverse reactions, and the even more exciting possibility of design-

ing complexing agents as carriers to enhance the absorption and thereby the pharmacologic activity of drugs that are otherwise poorly absorbed and inactive

REFERENCES

Anello, J A & G Levy Effect of complex formation on drug absorption X Effect of polysorbate 80 on the permeability of biologic membranes *J Pharm Sci* 1969 58, 721

Diamond, L. K, J L Naiman, D M Allen & F A Oski The treatment of iron-deficiency anemia palatable but ineffective iron medication *Pediatrics* 1963 31 1041

Hayton, W L, D E Guttman & G Levy Enhancement of steroid absorption by dialkylamides *J Pharm Sci* 1970, 59, 575

Kunin, C. M & M Finland Clinical pharmacology of the tetracycline antibiotics *Clin Pharmacol Therap* 1961, 2, 51

Levy, G & R H Reuning Effect of complex formation on drug absorption I Complexes of salicylic acid with absorbable and nonabsorbable compounds *J Pharm Sci* 1964, 53, 1471

Levy, G & S P Gucinski Studies on biologic membrane permeation kinetics and acute toxicity of drugs by means of goldfish *J Pharmacol Exp Therap* 1964 146, 80

Levy, G & T Matsuzawa Effect of complex formation on drug absorption II Lipoid soluble dye complexes *J Pharm Sci* 1965, 54, 1003

Levy, G, K E Miller & R H Reuning Effect of complex formation on drug absorption III The concentration and drug dependent effect of a nonionic surfactant *J Pharm Sci* 1966, 55, 394

Levy, G & J A Anello Effect of complex formation on drug absorption V Studies on the mechanism of the secobarbital absorption enhancing effect of polysorbate 80 in goldfish *J Pharm Sci* 1968, 57, 101

Levy G & E Mroszczak Effect of complex formation on drug absorption VI Drug permeation through an artificial lipoid barrier *J Pharm Sci* 1968 57 235

Levy G & A Perala Effect of polysorbate 80 and oleic acid on drug absorption from the rat intestine *J Pharm Sci* 1971 59, 874

Martin, A N, J Swarbrick & A Cammarata *Physical pharmacy* Lea & Fibiger Philadelphia, 2nd ed 1969, chapter 13

Reuning, R H & G Levy Effect of complex formation on drug absorption IV Role of intra gastric diffusion in the absorption of free and caffeine complexing salicylic acid from the rat stomach *J Pharm Sci* 1967, 56 843

Reuning, R H & G Levy Effect of complex formation on drug absorption VII Effect of complexation and self association on the absorption of caffeine *J Pharm Sci* 1968a, 57, 1335

Reuning R. H. & G Levy Effect of complex formation on drug absorption VIII Intestinal transfer characteristics of the salicylamide caffeine system *J Pharm Sci* 1968b 57, 1342

Reuning, R. H & G Levy Characterization of complex formation between small molecules by membrane permeation measurements *J Pharm Sci* 1968c 57 1556

Reuning, R H & G Levy Effect of complex formation on drug absorption IX Determination of the intestinal transfer rate constant of the salicylamide-caffeine complex. *J Pharm Sci* 1969, *58*, 79

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Importance of Pharmaceutical Formulation for Drug Absorption

By

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Interest in the influence of dosage form on drug absorption has mainly been focused on two aspects One is the possibility of modifying the absorption pattern in order to get an improved action of the drug by suitable pharmaceutical formulation The other is the risk of changing the absorption or the effect of a drug unintentionally The influence of the pharmaceutical formulation generally depends upon changes in the dissolution properties or in the amount of drug which is released for absorption Table 1 summarizes the formulation factors which affect the absorption of a drug

Improvements of drug action by pharmaceutical means

The *rate of absorption* for many drugs is limited by the rate of dissolution and in such cases it is possible to get a more rapid onset of action by improvement of the formulation To achieve rapid dissolution the most soluble form of the drug should be used and it should be formulated to give a large interface between drug and fluids

Fig 1 shows that pentobarbital (mebumalum NFN) sodium salt is absorbed quicker than the acid which is much less soluble

Another example is given in fig 2 A readily soluble salt of acetylsalicylic acid is absorbed more rapidly than the acid Because of the limited stability of acetylsalicylates, the salts are seldom used but instead the acid is combined with buffering substances In this way is it possible to get a salt formation *in situ* and increase the rate of absorption

By improving the dissolution it is sometimes possible to *increase the availability* of incompletely absorbed drugs This can be accomplished by the use of soluble salts, polymorphic forms with higher solubility or by increasing the effective surface

Suitable pharmaceutical formulation may also *reduce side-effects* of drugs Enteric coating and sustained release products are commonly used to reduce gastro-intestinal irritation

Table I

Formulation factors influencing the absorption of a drug

Solubility	e g	salt formation polymorphism solvates
Effective surface	e g.	particle size wetting disintegration of tablets etc
Chemical or physical interactions	e g	complex formation adsorption degradation
Physiological interactions	e g	absorption inhibitors absorption promotors

Prolongation of drug action is another important aim of pharmaceutical formulation The drug is supplied in a form which does not release all the dose immediately but continually over a certain time. Slow release of the

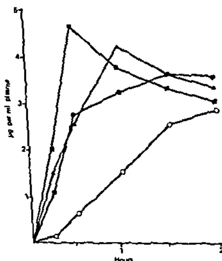


Fig 1 Pentobarbital concentration in plasma after administration of pentobarbital and pentobarbital sodium in capsules and in aqueous solution or suspension. Each curve represents the mean of two subjects

○ Pentobarbital capsules 200 mg

● Pentobarbital sodium capsules 220 mg

▲ Pentobarbital suspension 200 mg

■ Pentobarbital sodium solution 220 mg

(From SJÖGREN *et al.* 1965)

From the Pharmaceutical Laboratory, AB Hassle, Goteborg Sweden

Importance of Pharmaceutical Formulation for Drug Absorption

By

John Sjogren

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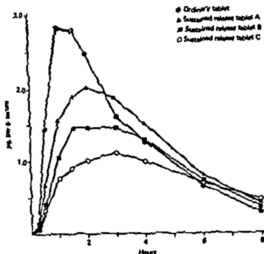


Fig. 4 Serum concentration of alprenolol and metabolites after administration in ordinary tablets and in three sustained release tablets. Mean values of a cross-over study in four subjects.

- ● Ordinary tablet
- ▲ Sustained release tablet A
- ■ Sustained release tablet B
- ○ Sustained release tablet C

(From JOHANSSON *et al* 1971)

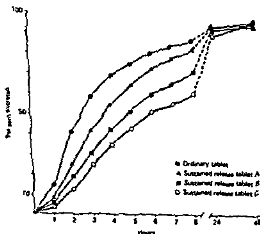


Fig 5 Per cent of the dose excreted in the urine after administration of alprenolol HCl in ordinary tablets and in three sustained release tablets. Mean values of a cross-over study in four subjects.

- ● Ordinary tablet
- ▲ Sustained release tablet A
- ■ Sustained release tablet B
- ○ Sustained release tablet C

(From JOHANSSON *et al* 1971).

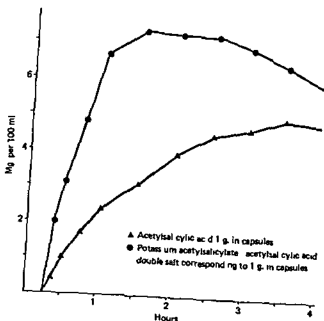


Fig 2 Salicylate concentration in plasma after administration of acetylsalicylic acid and potassium acetylsalicylate – acetylsalicylic acid double salt in capsules Mean values of a cross over study *in three subjects*

▲ Acetylsalicylic acid 1 g

● Potassium acetylsalicylate – acetylsalicylic acid equivalent to 1 g

drug can be achieved by the use of drug salts or complexes with sufficiently low solubility and this principle is used mainly in *parenteral* depot preparations In products for oral administration the release is generally controlled by a reduced effective surface or by the use of diffusion *processes*

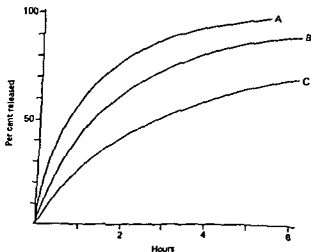


Fig 3 *In vitro* release of alprenolol HCl (aptin®) from three sustained release tablets (From JOHANSSON *et al* 1971)

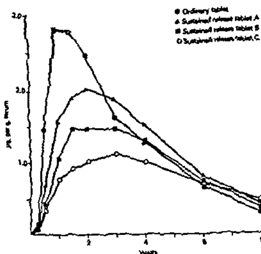


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(From JOHANSSON *et al* 1971)

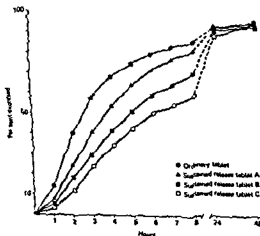


Fig. 5 Per cent of the dose excreted in the urine after administration of *alprenolol HCl* in ordinary tablets and in three *sustained release tablets* Mean values of a cross-over study in four subjects

- ● Ordinary tablet
- ▲ Sustained release tablet A
- ■ Sustained release tablet B
- ○ Sustained release tablet C

(From JOHANSSON *et al.* 1971)

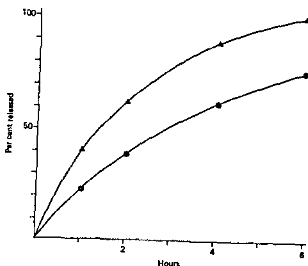


Fig 6 *In vitro* release of lithium sulphate from two sustained release tablets (From AMDISEN & SJOGREN 1968)

An example of how a deliberately reduced dissolution rate changes the absorption, blood levels and excretion of a drug is given in the following figures Alprenolol hydrochloride was formulated into sustained release tablets (duretter®) with different rates of release as shown in fig 3 By using a tritium labelled substance we followed the serum levels of radio activity (fig 4) and it is easily seen that the rate of absorption was closely correlated to the release *in vitro* The slower dissolution the less were the variations in the serum levels over the period studied Fig 5 shows that the excretion in urine was also closely correlated to the rate of release

Table 2

Per cent lithium recovered in the urine after oral administration of lithium sulphate in ordinary tablets and in the sustained release tablets of fig 6

Subject	Sust release tablet ●	Sust release tablet ▲	Rapidly disintegrating tablet
JS	95	96	92
KK	63	91	91
TEJ	75	95	92
SC	69	81	92
GE	71	93	87
Mean	74	91	91

(From AMDISEN & SJOGREN 1968)

If the release of the drug from the dosage form is too slow, however, the availability may be decreased and it is therefore always necessary to investigate sustained release products in this respect Reduced side effects attained by administration in sustained release form may otherwise be obtained at the cost of lower absorption When we developed a sustained release lithium tablet we investigated the effect of the release rates on the availability

The slower of two compositions in fig 6 was not completely available as seen in table 2 *This tablet can consequently be expected to require a higher* daily dose than ordinary tablets in order to maintain the same average serum levels The other composition which was fully available proved to give the same average serum level under steady state conditions as seen in fig 7 We can also see that the fluctuations in the serum concentration were less after the sustained release tablets twice a day than after ordinary tablets three times a day

Therapeutic inequivalence

Today it is well known that the same amount of substance given in different dosage forms or in different brands of the same dosage form does not

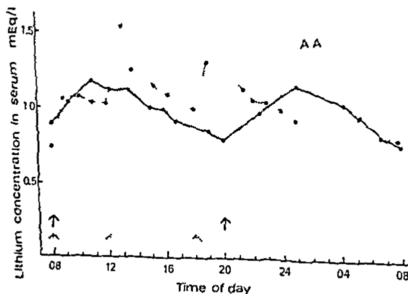


Fig. 7 Lithium levels in serum under steady state conditions on 37 meq per day
—— Lithium sulphate sustained release tablets (lithionit duretter®) twice a day
Lithium carbonate tablets three times a day
(From AMDISEN & SJÖGREN 1968)

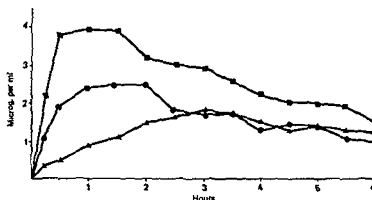


Fig 8 Secobarbital concentration in plasma after administration of rapidly disintegrating tablets

■ Secobarbital sodium 2 × 100 mg
▲ Secobarbital granulated with alcohol 2 × 91 mg
● Secobarbital granulated with 20 % gelatine solution 2 × 91 mg
(J Sjögren & L Sölvell, unpublished results)

always give the same clinical results This type of formulation effect occurs unintentionally and will therefore often be a complete surprise for both the producer and the prescribing physician Biopharmaceutical discrepancies between different products are due to variations in the four main factors of table 1

It might appear surprising that unexpected inequivalence has occurred due to different solubility of the active substance but if you do not check which polymorph you use in your product it might easily happen Very few drug monographs have tests on polymorphism The soluble salts of many acid drugs such as barbiturates, acetylsalicylic acid, present technical problems in tablet production and if you are not aware of the differences in absorption rate you will of course use the form of the drug which it technically most suitable

When formulating a drug substance into a pharmaceutical preparation it is generally necessary to add other substances and very often also to bind the initial drug particles together in bigger aggregates This will change the dissolution properties of the drug by altering the surface or by interactions of various kinds Sometimes this will change the absorption of the drug sufficiently to be of clinical importance

There are no simple laboratory test methods available which can be used generally for biopharmaceutical evaluation of different products The disintegration test which is traditionally applied to tablets and capsules is sometimes misleading Dissolution tests give us better possibilities of predicting the *in vivo* properties but not even these tests can be expected to be generally

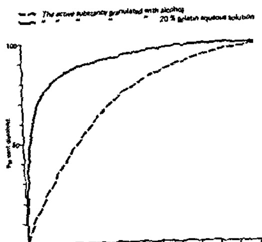


Fig 9 *In vitro* dissolution of the same two secorbarbital tablets as in fig 8 (Beaker method propeller agitation 150 r p m)
- - - the active substance granulated with alcohol
—— the active substance granulated with 20 % gelatine aqueous solution

applicable In order to use *in vitro* techniques for biopharmaceutical evaluation it is necessary to adjust the method to the particular product and to assess the results with a good measure of common sense

Let me then show you some examples of problems which we have encountered in the development of pharmaceutical products Fig. 8 gives the

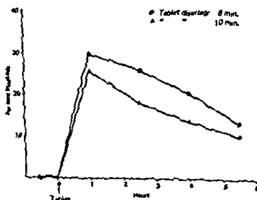


Fig. 10 Blockade of work induced increase in heart rate after administration of 100 mg alprenolol HCl in tablets with different rates of disintegration. Mean values of cross-over studies in four subjects.
● disintegration time (BP 68) 6 minutes
▲ disintegration time (BP 68) 10 minutes
(G Johnsson & J Sjögren, *unpublished results*)

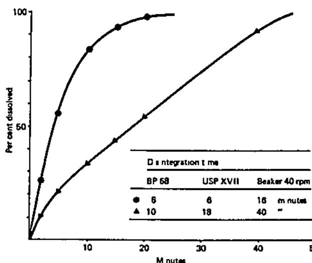


Fig 11 *In vitro* dissolution in water of the two alprenolol HCl tablets from fig 10 Beaker method with propeller agitation at 40 r p m
The disintegration times according to three methods are given in the inserted table

plasma levels of secobarbital from tablets with very rapid disintegration (approximately 10 seconds) The rapid absorption of the sodium salt is in agreement with its higher solubility The difference between the two secobarbital tablets is more surprising, however, since the two compositions

Table 3

Effect of some common additives on the absorption of iron from ferrous sulphate solutions Mean values from cross over studies in healthy subjects

Additive	Amount	Number of subjects	Absorption ratio with/ without additive
Citric acid	192 mg	5	0 62
Ascorbic acid	200 mg	13	1 33
Fumaric acid	116 mg	11	1 08
Succinic acid	120 mg	12	1 45
Mannitol	4 g	4	1 74
Sorbitol	4 g	4	1 41
Sorbitol	8 g	4	1 62
Sorbitol	14 g	4	2 71
Sucrose	4 g	4	1 02
Fructose	4 g	4	1 03
Lactose	4 g	4	0 85
Glucose	14 g	4	0 78

(From Brise & Hallberg 1962a & b, and Hallberg *et al* 1966)

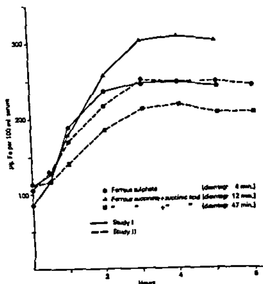


Fig 12 *Serum iron concentration* after administration of tablets containing 75 mg Fe^{++} Mean values of cross-over studies in five subjects.

● Ferrous sulphate (disintegration 4 min)
▲ Ferrous succinate + succinic acid 220 mg (disintegration 12 min)
■ Ferrous succinate + succinic acid 220 mg (disintegration 47 min)
——— Series I
- - - - - Series II
(J SJÖGREN & L. SOLVELL, unpublished results)

are identical with the exception of the gelatine content in one of them Granulation of secobarbital powder with an aqueous gelatine solution binds the particles together into granules but the particles are not soluble in water and they retain their individual shape within the granules Granulating the barbiturate with alcohol results in more compact aggregates because alcohol dissolves the initial drug particles and granules with a smaller effective surface are formed Fig 9 shows that the alcohol granulated tablet also dissolved much slower *in vitro* than the other tablet.

Most problems reported regarding therepeutic inequivalence concern drugs with low solubility but soluble drugs may also give problems Fig 10 shows a recent example where we compared two formulas of aptin® tablets with slightly different disintegration The active substance, alprenolol hydrochloride is very soluble in water We were surprised that such a small difference in the tablet disintegration could reduce the availability Therefore we also investigated the dissolution and disintegration with other test methods As

you can see from fig 11, the differences between the formulas were greater at lower agitation but still not big enough to explain the *in vivo* results

The absorption of iron from the gastro intestinal tract can be influenced by a lot of factors In table 3 I have compiled the effect on the absorption of some addivites which are commonly used in iron products e g syrups The amount of iron which a patient absorbs from a product containing glucose and citric acid cannot be expected to be as high as from a product containing sorbitol and ascorbic acid as adjuvants even if the iron content and the iron salt are the same in both products

We have used the absorption promoting effect of succinic acid to improve the utilization of iron from some of our products Fig 12 shows the effect on serum iron concentration of two experimental tablet formulas containing ferrous succinate The first one gave higher concentration of iron than the reference ferrous sulphate tablet did whereas the other gave lower The rate of dissolution in dilute acid followed the disintegration closely For this formula rapid disintegration was obviously necessary to get a good absorption Another formula, a sugar coated tablet containing ferrous

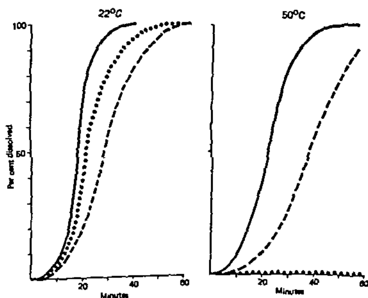


Fig 13 *In vitro* dissolution of iron in 0 05 N HCl from sugar coated tablets containing ferrous sulphate and succinic acid after one year s storage

Beaker method 250 r p m propeller agitation

– – BP 63 disintegration test

—— BP 63 disintegration test with guided disc

(G EKENVED T E. JONSSON & L. SOLVELL, unpublished results)

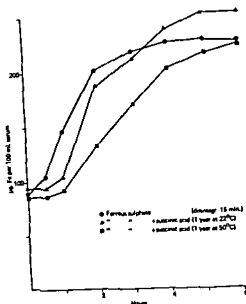


Fig 14 Serum iron concentration after administration of tablets containing 75 mg Fe^{++}
Mean values of a cross-over study in six subjects

● Ferrous sulphate (disintegration 15 min)
▲ Ferrous sulphate + succinic acid 370 mg (stored at 22°)
■ Ferrous sulphate + succinic acid 370 mg (stored at 50°)
(G EKENVED, T E JONSSON & L SÖLVELL, unpublished results)

sulphate and succinic acid, required approximately half an hour to dissolve After 1 year's storage at elevated temperature it did not dissolve at all (fig 13) in our dissolution test because the sugar coating did not dissolve completely but left a tough film surrounding the tablet. In the disintegration testing apparatus these tablets dissolved although slower than the other *In vivo* studies (fig 14) revealed that *in this case the beaker method was not suitable* for predicting the absorption The tablet stored at high temperature was absorbed more slowly than the other but the availability did not appear to be significantly changed. The latter two examples demonstrate the difficulty in interpreting *in vitro* data. One product which dissolved in less than 1 hour was not fully available while another with still slower dissolution was

With these examples I have tried to illustrate some ways by which the pharmaceutical formulation may influence the absorption of drugs I have also tried to give examples of the difficultes which are encountered in the development of new products It is obvious that the development of pharmaceutical products in the future will require much more biopharmaceutical

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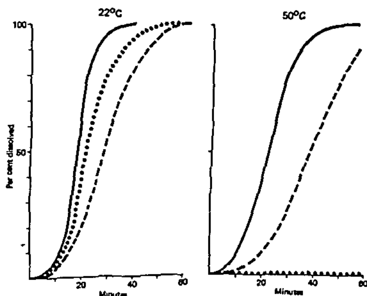


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From the Galenical Research Department of F Hoffmann La Roche & Co., Ltd, Basle, Switzerland

The Desorption of Medicinal Substances from Adsorbents in Oral Pharmaceutical Suspensions

By

Kurt Munzel

Abstract In children the desorption of diazepam from an adsorbate in a suspension syrup and hence its biovailability is retarded as compared to that of a drop solution, although the anti pentetrazol test in mice could not reveal such a difference between these two dosage forms

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Oral suspensions are complicated disperse systems, the physical stability of which – no settling or floating resp creaming of the solid particles – has to be approached mainly empirically STOKES' law which indicates the role of the diameter of the particle, of the difference between the density of the solid and the liquid phase, and of the viscosity of the latter, is of little value for practical formulation work.

STOKES' law

$$v = \frac{d^2 (\varrho_P - \varrho_M)\ g}{18\,\eta}$$

v = velocity of settling [cm/sec]
d = diameter of the particle [cm]
ϱ_P = density of the particle [g/cm³]
ϱ_M = density of the medium [g/cm³]
g = gravitational constant [981 cm/sec²]
η = viscosity of the medium (in P)

HIGUCHI s equation (1968) stresses the specific surface area of the powder and the prorosity of the powder bed as further parameters

HIGUCHI's equation

$$\mu = \frac{(\varrho_P - \varrho_M)\,g}{k\ \eta\ S^2} \cdot \frac{\varepsilon^3}{1 - \varepsilon}$$

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investigation than it has to date The pharmaceutical manufacturer must have facilities to study e g pharmacodynamics and pharmacokinetics in man to be able to develop reliable pharmaceutical products

REFERENCES

Amdisen, A & J Sjogren Lithium absorption from sustained release tablets (duretter®) *Acta Pharmaceutica Suec* 1968, **5**, 465–472.

Brise, H & L Hallberg Effect of ascorbic acid on iron absorption *Acta Med Scand* 1962a, **171**, Suppl 376, 51–58

Brise, H & L Hallberg Effect of succinic acid on iron absorption *Acta Med Scan* 1962b **171**, Suppl 376, 59–73

Hallberg, L., L Solvell & H Brise Search for substances promoting the absorption of iron *Acta Med Scand* 1966, Suppl 459, 11–23

Johansson, R, C G Regårdh & J Sjogren Absorption of alprenolol in man from tablets with different rates of release *Acta Pharm Suecica* 1971, **8**, 59–70

Sjogren, J, L. Solvell & I Karlsson Studies on the absorption rate of barbiturates in man *Acta Med Scand* 1965, **178**, 553–559

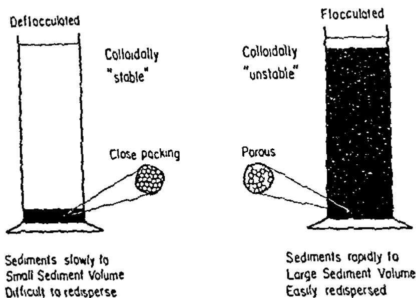
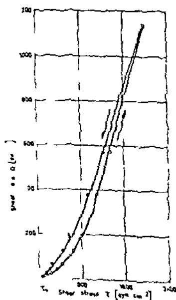


Fig 2. Flocculated and unflocculated suspensions (HIESTAND 1964)



[illegible] d is 80 dyn cm⁻² [illegible] 1964)

μ = flow velocity
k = the KOZENY constant (~ 5)
η = the viscosity of the external fluid
S = specific surface area of the powder
ε = porosity of the powder bed (volume fraction of the spaces between the particles filled with liquid and eventually air)
$1 - \varepsilon$ = volume fraction of the powder particles of the bed

The formulator of an oral suspension should attempt

- to provide a suitable low practical yield value τ_0 for the product (fig 1) which, however, should not prevent an immediate flow of the suspension on pouring out of the bottle and
- to build up a flocculated structure of the solid particles (fig 2)

A suitable thixotropy of the product (fig 3) is favourable to its physical stabilisation A slight tendency to settling of an oral suspension does not prevent its use, if it can readily be reversed by gentle shaking

Suspended oral dosage forms are of advantage mainly in paediatric or geriatric medicine for the administration of insoluble or slightly soluble drugs in liquid form for internal use

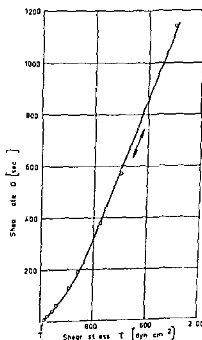


Fig 1 Rheogram of a pseudoplastic suspension with a low practical yield value τ τ_0 was determined by means of the Visco Balance (MÜNZEL & BERNEIS 1964) and is 70 dyn cm^{-2}

Table I

Formulations of diazepam syrups I, II and III, amount of diazepam in mg/kg mice, administered in the form of 4 different preparations, to obtain an APR = 2.0

Formula	Ingredients			Diazepam in mg/kg mice per os to obtain an APR = 2 0
	Adsorbate	Suspension stabiliser	Surfactant	
Syrup I	*Diazepam* + veegum S-6814*	Tragacanth of high viscosity	Nonionic surfactant	1.8
Syrup II	Diazepam + veegum S-6814*	Veegum K,** *Sodium carboxy* methylcellulose (CMC)	*Nonionic* surfactant	4 0
Syrup III	Diazepam + veegum S-6814*	Avicel RC-501***	—	10 0
Drop solution of diazepam	—	—	—	1 7

* Inorganic complex colloidal magnesium aluminium silicate
** *Inorganic colloidal magnesium aluminium silicate, primarily useful for pharma*ceutical *acidic* suspensions, has a high acid compatibility
*** *Microcrystalline cellulose with 11 % CMC, Manufacturer* FMC *Corporation,* Avicel International Marcus Hook, P A. 19061 (U S A)

It can be clearly seen that only syrup I exhibits in mice a diazepam dose *for an* APR *2 0 similar to that of the drop solution* The speed of the desorption of diazepam from the adsorbate is obviously the highest in syrup I In syrup II the additional amount of veegum K as a gel producing agent seems to decrease the rate of desorption and, therefore, to increase the mg/kg dose in mice, in syrup III, however, the Avicel RC-501 and the lacking surfactant exert the same phenomenon to an even enhanced extent. Since Avicel RC 501 in syrup III contains also CMC which is used in syrup II, a flocculation of CMC in the acidic gastric fluid is possible and may further retard desorption

Thereafter, blood levels of diazepam were determined in a cross-over study in 6 children after a single oral *administration of 0 36 mg/kg* in the two dosage forms drop solution and syrup I (VIALA & CANO 1968) (fig. 4) The blood-assay procedure was based on the use of gas liquid chromatography after extraction of diazepam and its hydrolysis in methylamino-

In general, in suspensions a slightly soluble active compound should not be adsorbed at a solid interface, unless

- the taste is very unpleasant, even with the small amounts dissolved, and
- the chemical stability has to be improved resp the instability to be reduced by adsorption

Adsorption is a phenomenon of surface effect where the added molecules are partitioned preferentially at an interface

Material and Methods

In this paper we are dealing with the adsorption of the drug diazepam on clays, diazepam has a solubility of about 6 mg/100 ml at room temperature Even at this low concentration it possesses an extremely unpleasant taste which requires adsorption on a *carrier substance which is in this case veegum® (manufacturer* R T VANDERBILT Company, New York), an inorganic complex colloidal Magnesium Aluminium Silicate. Such a physical adsorption, due to VAN DER WAALS forces, is reversible, the removal of the adsorbate from the adsorbent being known as *desorption* In pharmaceutical suspensions, however, desorption should not occur during the shelf life of the product, but only in the gastrointestinal tract after oral administration under the influence of the gastric hydrochloric acid, the bile salts, the pH, the ionic strength of the gastrointestinal content etc Desorption is an absorption rate limiting process and may therefore be considered to become a biopharmaceutical problem The aim of this study was to investigate whether the physiological availability of diazepam from different suspension formulas containing an adsorbate of diazepam on veegum® is advisable or not compared to that of a drop solution (marketed only in France) which, unfortunately, possesses a very bitter taste

For the preliminary evaluation of the activity of different diazepam preparations in animals, the antagonism of diazepam to the convulsive effect of an intravenous administration of pentetrazol (cardiazol®) in mice may be used (Test method elaborated by Dr J Blum, Medical Department of F Hoffman La Roche & Co, Ltd, Basle. The detailed procedure will be published later in a pharmacological periodical) Pentetrazol is given by infusion until a tonic extension of the hind limbs of the mice occurs without resp 30 minutes after the oral administration of an anticonvulsive drug, the ratio of these two pentetrazol doses (anti pentetrazol ratio = APR) is calculated As a limit for an effective preparation the ratio of 2 has been arbitrarily chosen The equi effective doses refer to this value of 2 All drugs with an APR of 2 in a suitable dose, or with an APR of even > 2 are considered to be anticonvulsive In speaking of equi effective doses of the APR we mean those doses which have the same antagonistic effect against penetetrazol convulsions, i e 30 minutes after the oral administration of the dose of an anticonvulsant in mg/kg mice, the double quantity of pentetrazol is needed to produce convulsions compared with the quantity required without drug If there is only one active ingredient, e g diazepam, but in different formulations, the mg/kg mice mean the amount of the drug in the form of the preparation in question

Results

Table 1 indicates the different formulations of diazepam syrups and the required amount of diazepam in mg/kg mice for an APR = 2

REFERENCES

Higuchi, T *J Amer Pharm Ass* 1958, 47, 657

Munzel, K. & K. Berneis *Pharm Acta Helv* 1964, 39, 88

Hiestand, E. N *J Pharm Sci* 1964, 53, 10

Viala, A & J P Cano *Teneurs sanguines en diazepam ("VALIUM" N P)* après administration du medicament sous forme de gouttes ou de sirop *Therapie* 1968, 23, 775

Cano, J P, L. Vignoli & A. Viala Determination du diazepam dans le sang et l urine par chromatographie en phase aqueuse *Annal Pharm Franç* 1967, 25, 697, 821

Cano, J P *Contribution a l'etude pharmacologique et toxicologique du diazepam ("VALIUM" N P) et de quelques benzodiazepines (application de la chromatographie en phase gazeuse)* Thèse Doct Pharm (Etat), Marseille, 13 mai 1969

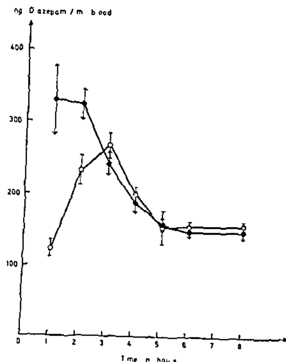


Fig 4 Blood levels of diazepam after administration of 0 36 mg/kg as determined in 6 children in a cross over trial (VIALA & CANO 1968) in the form of the
●——● drop solution and
○——○ syrup I (table 1)
(Mean and standard error of the mean)

chloro benzophenone (CANO *et al* 1967, CANO 1969) Diazepam is definitely more rapidly absorbed in solution than in syrup and the peak is higher with the solution The peak of the solution curve is reached in one hour, that of the syrup curve in 3 hours The N-demethylated metabolite, which is determined by a similar technique, can be detected 5–6 hours after drop solution or syrup medication and remains in the blood more than one week Hence, in summary, the desorption of diazepam in humans from an adsorbate in a suspension syrup is an absorption limiting process, although the preliminary anti-pentetrazol-test in mice could not clearly reveal such a difference between the drop solution and the suspension syrup I

Acknowledgements

We are indebted to Dr J Blum and his staff, Medical Department of F Hoffmann-La Roche & Co , Ltd , Basle, for their assistance in performing the anti-pentetrazol-test in mice

form a slurry Stir the slurry with a spatula until the pancreatin dissolves Filter through a 25 cm diameter Buchner funnel and wash the filter cake with three 200 ml portions of distilled water Dilute the filtrate to 2 l with distilled water and store at 5° (stable up to 48 hours) Add 400 ml of the pancreatin solution (equivalent to 5 g of 4X pancreatin) and 20 0 g pH 7.2 phosphate buffer powder (Hartman Leddon Co., Inc. Philadelphia, Pa) to 19 l distilled water After the buffer salt dissolves, adjust the final volume to 20 l with distilled water

Subjects

A *Diazoxide*

We used four hypertensive subjects (CALESNICK *et al* 1965) who were previously under continuous observation and treatment for varying degrees of moderate to severe hypertension for more than two years. Observations were made while the subject was in a continuous fasting state and were started between 10 00 a.m. and 12 00 noon No anti hypertensive drugs were administered for two weeks prior to the study

B *Griseofulvin*

Ten normal subjects were used in the first experiment (KATCHEN & SYMCHOWICZ 1967), eight, in the second (SYMCHOWICZ & KATCHEN 1968), and eighteen, in the third (SYMCHOWICZ & KATCHEN 1968) Each subject took 500 mg griseofulvin before breakfast at approximately 8 00 a m. and ate a standard meal at 10 00 a m. All subjects were told to avoid the foods and drugs known to interfere with the griseofulvin plasma assay for the next 24 hours.

Methods

A. *Dissolution rate*

Add 20 liters 0 1 N HCl or simulated intestinal fluid to a 10" × 18" pyrex cylinder and maintain at 37 ± 1° Place the tablet or capsule to be assayed in a stainless steel cylinder (25 × 64 mm) covered at each end with a 40-mesh sthainless steel screen. Move the stainless steel cylinder in a vertical plane beneath the surface of the test fluid with a Stoll-Gershberg apparatus. Solutions or dispersions are added directly to the test fluid by following them to flow from a volumetric pipette held midway between the stirrer and the wall of the pyrex cylinder The test fluid is stirred continuously with a four blade turbine type propeller at 240–260 r p.m At suitable intervals remove a small sample of fluid for spectrophotometric determination of dissolved drug

B *Plasma assays*

Griseofulvin was assayed fluorometrically (WEINSTEIN & BLANK 1960) and diazoxide spectrophotometrically (SYMCHOWICZ *et al* 1967)

C *Absorption*

Calculate area under the blood level curve by the trapezoidal rule Divide area by time interval covered to obtain mean plasma level, our absorption index

Experimental design

A. *Diazoxide*

Each subject received 600 mg diazoxide (40 ml of the sterile solution, six capsules or six tablets. In a repeat measure design, we gave each subject single doses of as

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Correlation of Dissolution Rate and Drug Absorption in Man

By

Bernard Katchen

Abstract Diazoxide blood levels in man were measured after single 600 mg oral doses in solution, capsule and tablet form. A high correlation was found between dissolution rate in 0.1 N-HCl and area under the 0–6 hour blood level curve. Griseofulvin absorption from 10 different preparations was studied in man. Plasma levels were followed for 1 day after a single oral 500 mg dose, and for 7 days after daily 500 mg doses. A high correlation was found between dissolution rate in simulated intestinal fluid and area under the 0–25, 49–173, and 0–173 hour plasma level curves.

Key words Griseofulvin – diazoxide – intestinal absorption

Drug absorption studies in man are expensive and time consuming. Consequently, a simple, cheap and rapid *in vitro* method that predicts bioavailability would be highly desirable. Dissolution rate offers this promise, but suffers from the drawback that it must be correlated with *in vivo* data before it will yield quantitative predictions. This paper summarizes our dissolution rate and oral absorption studies in man with diazoxide (hyperstat®) and griseofulvin.

Experimental

Materials

A *Dosage forms*

1 Sterile solution of sodium diazoxide containing the equivalent of 300 mg diazoxide in 20 ml water adjusted to pH 11.4 with NaOH.

2 Hard gelatin capsules containing the equivalent of 100 mg diazoxide as sodium diazoxide.

3 Uncoated compressed tablets containing 100 mg diazoxide.

4 Three different 125 mg griseofulvin capsules and seven different griseofulvin tablets.

B *Simulated intestinal fluid*

To 200 g solka floc (BW-100, Brown Co., Boston, Mass.) add 25 g 4X N.F. pancreatin (Viokase, Viobin Corp., Monticello, Illinois) and enough distilled water to

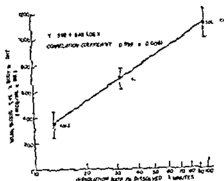


Fig 1 Correlation of dissolution rate and mean diazoxide blood level Brackets enclose 95 % confidence intervals of regression line

preparation clogged the 40-mesh screen of the sample holder, we could not correlate its dissolution rate with mean plasma level

In distilled water, the dissolution rates of preparations 1, 2, and 3 are very low and cannot be differentiated. However, in simulated intestinal fluid except for preparation 1, the dissolution rates are much higher and reveal differences between preparations related to their formulations Because each preparation could not be characterized with a single dissolution rate contsant, we used amount dissolved in a given time as a measure of dissolution rate A meaningful correlation between mean plasma level and dissolution rates in distilled water could not be found because most of the griseofulvin preparations have low dissolution rates in this solvent However a good correlation was found between mean griseofulvin plasma level and dissolution rate in simulated intestinal fluid The best correlation

Table 2

Experiment I *In vitro* dissolution rate and griseofulvin absorption in man

Preparation	Mean plasma level* (μg ml)	% dissolved in simulated intestinal fluid *Minutes* 5	10	15	30	60	% dissolved in distilled water *Minutes* 5	10	15	30	60
1	0.34	6	9	11	13	—	4	6	9	13	22
2	0 65	7	15	24	35	52	4	6	7	11	16
3	0 73	3	12	26	56	75	0	2	5	10	15
4	0 90	0	27	69	96	100	0	0	34	93	100

* (Area under the 0–24 hr plasma level curve) 24

many of the different preparations as was feasible, at intervals of at least 10 days. Blood samples were taken just prior to, and up to 6 hours after administration of the drug

B *Griseofulvin*

1 *Experiment I* At one week intervals, we gave preparations 1–5 to ten humans in a three factor repeat measure design (WINER 1962a) We drew blood at 0, 2, 4, 8, and 24 hours postdrug

2 *Experiment II* We gave preparations 2, 6, 7, and 8 at weekly intervals to eight normal humans in a replicated Latin square crossover design (WINER 1962b) Before breakfast, each subject ingested one 500 mg tablet (preparation 7) or four 125 mg tablets (preparations 2, 6, and 8)

3 *Experiment III* Three different griseofulvin preparations (7, 9, 10) were given to 18 normal subjects Each subject ingested one 500 mg tablet (preparation 7) or two 250 mg tablets (preparations 9 and 10) Preparation 9 has the same composition as 8 and preparation 10, the same composition as 2

Results and Discussion

I Diazoxide

Table 1 shows the mean plasma levels (normalized for distribution volume) and dissolution rates of each preparation Because of the small number of subjects in this study and the large spread in body weights (50–104 kg), normalizing the data for body weight (distribution volume) is essential for a high correlation between dissolution rate and oral absorption (fig 1)

II Griseofulvin

Table 2 shows the mean 24 hour plasma levels and dissolution rates of the first 4 preparations in Experiment I Because the contents of the fifth

Table 1

Dissolution rates of diazoxide dosage forms and absorption in man

Dosage form	Mean plasma level* (µg/ml × kg)	% dissolved Minutes 5	10	15	30	60
Solution	1095	100	100	—	—	—
Capsule	688	3	20	32	68	83
Tablet	354	3	8	13	52	65

* [Area under the 0–6 hr plasma level curve] 6 × body weight

Table 4

Experiment III *In vitro* dissolution rate and griseofulvin absorption in man

Preparation	Mean griseofulvin plasma levels (μg/ml)*									% dissolved in simulated intestinal fluid				
	Hours									*Minutes*				
	0-25	25-49	49-53	53-97	97-101	101-169	169-173	49-173	0-173	5	10	15	30	60
7	0.87	0.83	1.42	1.44	1.54	1.54	1.54	1.50	1.31	17	30	37	50	65
9	1.03	1.05	1.57	1.64	1.72	1.67	1.58	1.65	1.48	21	48	70	94	100
10	0.76	0.84	1.40	1.42	1.47	1.43	1.32	1.42	1.24	11	17	22	33	44

* Areas under curve divided by periods as indicated

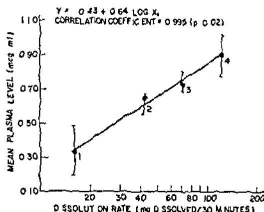


Fig 2 Experiment I Correlation of dissolution rate and mean griseofulvin plasma level Brackets enclose 95 % confidence intervals of line Preparation number beneath each point

Table 3

Experiment II *In vitro* dissolution rate and griseofulvin absorption in man

Preparation	Mean plasma level* (μg/ml)	% dissolved in simulated intestinal fluid Minutes 5	10	15	30	60
2	0 74	7	15	24	35	52
6	0 93	13	31	42	55	75
7	0 83	17	30	37	50	65
8	1 02	24	54	77	97	99

* [Area under the 0-25 hr plasma level curve] 25

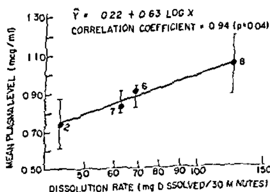


Fig 3 Experiment II Correlation of dissolution rate and mean griseofulvin plasma level Brackets enclose 95 % confidence intervals of line Preparation number next to each point

Table 4

Experiment III *In vitro* dissolution rate and griseofulvin absorption in man

Preparation	Mean griseofulvin plasma levels (μg/ml)*									% dissolved in simulated intestinal fluid				
	Hours									Minutes				
	0 25	25-49	49 53	53 97	97 101	101 169	169 173	49 173	0-173	5	10	15	30	60
7	0 87	0 83	1 42	1 44	1.54	1 54	1 54	1 50	1 31	17	30	37	50	65
9	1 03	1 05	1.57	1 64	1 72	1 67	1 58	1 65	1 48	21	48	70	94	100
10	0 76	0 84	1 40	1 42	1 47	1 43	1.32	1 42	1 24	11	17	22	33	44

* Areas under curve divided by periods as indicated

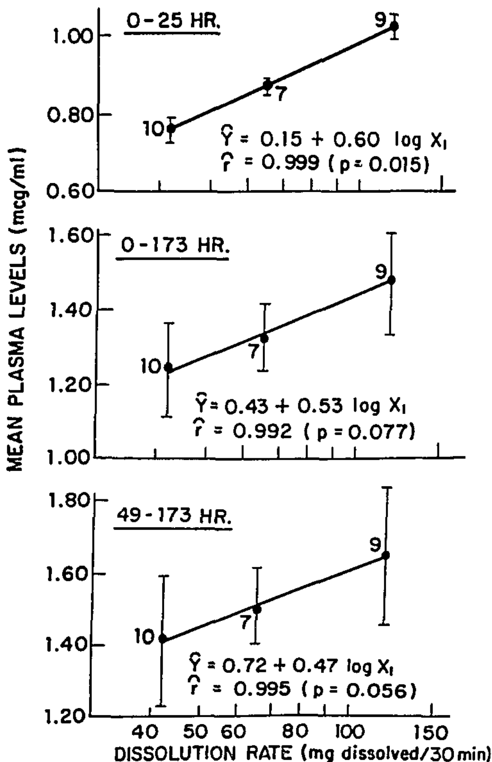


Fig 4 Experiment III Correlation of dissolution rate and mean griseofulvin plasma level Brackets enclose 95 % confidence intervals of line Preparation number next to each point

Table 5

Summary of regression equations for correlation between griseofulvin absorption and dissolution rate in simulated intestinal fluid.

Study	Time span (hours)	Least square regression equation	Correlation coefficient	P
I	0–24	$= -0.43 + 0.64 \log X_1$	0.995	0.02
II	0–25	$= -0.22 + 0.63 \log X_1$	0.94	0.04
III	0–25	$= 0.15 + 0.60 \log X_1$	0.999	0.015
III	0–173	$= 0.43 + 0.53 \log X_1$	0.992	0.077
III	49–173	$= 0.72 + 0.47 \log X_1$	0.995	0.056

is obtained with the logarithm of the 30 min dissolution values (fig 2). The correlation coefficient is 0.995 ($P = 0.02$). The 15 min dissolution values have a correlation coefficient of 0.96 ($P = 0.05$), the 10 min values, 0.82 ($P = 0.10$).

The high correlation coefficients in this study were obtained with preparations that differ widely in their composition and dissolution rates. This suggests that the increased plasma levels seen in man with various griseofulvin formulations (DUNCAN *et al.* 1962, ATKINSON *et al.* 1962, CROUNSE 1961) are due primarily to improved dissolution rates.

In Experiment II, we evaluated the reproducibility of the correlation with a new group of subjects and three new preparations. Preparation 2, which was retained as a standard (table 3), yields a 25 hr mean area (0.74) in good agreement with the 24 hr mean area in the first study (0.65). The regression equation (fig. 3) also compares favorably.

Table 6

Reproducibility of griseofulvin absorption (mean plasma level)

Preparation	Experiments I	II	III
2	0.65	0.74	0.76[a]
7		0.83	0.87
8		1.02	1.03[b]

[a] Preparation 10 but same composition as 2.
[b] Preparation 9 but same composition as 8.

In Experiment III, we studied the correlation between dissolution rate and drug absorption for 7 days, because griseofulvin therapy requires prolonged drug administration. Table 4 shows the mean areas under various segments of the plasma level curve. During the seven day span in which the drug was given once daily, good correlations are seen (fig. 4) after 25 hrs (effect of a single dose), 49–173 hrs (the plateau region), and 0–173 hrs (total experimental period). The correlation coefficients for the three time periods are 0.999 (P = 0.015), 0.992 (P = 0.077), and 0.995 (P = 0.056). The slope of the 0–25 hrs regression line compares favorably with the slope of the single dose treatment (0.60 in fig. 4 versus 0.63 in fig. 3). This would be expected because 9 and 10 have the same composition as 8 and 2, respectively, and preparation 7 is repeated (0.83 versus 0.87). The slopes for the later time intervals are lower than the 0–25 hrs slope because differences between the mean plasma levels of the preparations are smaller for the 49–173 hr period.

Table 5 summarizes the regression equations for all of the griseofulvin studies and table 6, the mean areas of preparations that were studied more than once. The data clearly show that the correlation between griseofulvin bioavailability and dissolution rate is reproducible and that the bioavailability of a given preparation is reproducible with different subject groups. Dissolution rates of griseofulvin dosage forms, therefore, should predict griseofulvin biovailability and greatly reduce the need for costly and time consuming studies in man.

Summary

1. Diazoxide absorption from tablet, capsule and liquid dosage forms was measured in man following single oral 600 mg doses. A high correlation was found between the area under the 0–6 hour blood level curve and dissolution rate in 0.1 N HCl.

2. Griseofulvin absorption from 3 capsules and 7 tablets was measured in man following single oral 500 mg doses. A highly reproducible correlation was found between the area under the 0–25 hour plasma level curve and dissolution rate in simulated intestinal fluid.

3. Griseofulvin absorption from 3 tablets was measured in man for 7 days after single daily oral 500 mg doses. A high correlation was found between dissolution rate in simulated intestinal fluid and area under the 0–25, 49–173 and 0–173 hour plasma level curves. The correlation for each time period yields a different regression equation.

REFERENCES

Atkinson, R. M, C. Bedford, K. J Child & E. G Tomich The effect of griseofulvin particle size on blood levels in man. *Antibiot Chemotherapy* 1962, 12, 232–238

Calesnick, B, B Katchen & J Black. Importance of dissolution rates in producing effective blood levels in man. *J Pharm Sci* 1965, 54, 1277–1280

Crounse R. G Human pharmacology of griseofulvin The effect of fat intake on gastrointestinal absorption. *J Invest Dermatol* 1961, 37, 529–533

Duncan W A M G McDonald & M J Thornton Some factors influencing the absorption of griseofulvin from the gastrointestinal tract. *J Pharm Pharmacol* 1962 14, 217–224

Katchen B & S Symchowicz Correlation of dissolution rate and griseofulvin absorption in man *J Pharm Sci* 1967, 56, 1108–1111

Symchowicz, S & B Katchen Griseofulvin absorption in man after single and repeated treatments and its correlation with dissolution rates *J Pharm Sci* 1968, 57, 1383–1386

Symchowicz, S L Winston, J Black, M Smith, B Calesnick & I I A. Tabachnick Diazoxide blood levels in man. *J Pharm Sci* 1967, 56, 912 914

Weinstein G D & H Blank Quantitative determination of griseofulvin by a spectrophotofluorometric assay *Arch Dermatol* 1960, 81, 746–749

Winer B J *Statistical principles in experimental design.* McGraw Hill Book Co, New York, 1962a page 319

Winer B J *Statistical principles in experimental design* McGraw Hill Book Co, New York, 1962b page 518

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The Gastrointestinal Absorption of Anticholinergic Drugs: Comparison between Individuals

By

Bjorn Beermann, Kjell Hellstrom and Anders Rosén

Key-words Atropine – parasympatholytics – intestinal absorption

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The ratio of parenteral to oral equipotent doses varies considerably among some anticholinergic drugs Whereas atropine appears to be of equal potency irrespective of the route of administration the oral dose of propantheline and methylscopolamine has to exceed the parenteral one by a factor of 10 and 100 respectively before equal anticholinergic effects are obtained (MOLLER & ROSÉN 1968)

Studies with intestinal loops in intact rats (LEVINE & PELICAN 1964) have demonstrated considerable differences in the absorption of several anticholinergic drugs To investigate whether a poor absorption might be responsible for the low efficacy of some orally administered drugs we have adopted a radiochemical technique The present report summarizes the results so far obtained

Materials and Methods

Experimental procedure Healthy male subjects 40–60 years of age were provided with a nasal double lumen tube that was allowed to pass to the desired levels of the intestine before the start of the experiment The test solution which was administered in the morning, was made up of 1) tritiated material of the drug 2) non labelled material of the drug in amounts which – with the exception of butylscopolamine – in general produced anticholinergic effects, 3) the non absorbable marker polyethylene glycol (PEG, 5 g) and 4) water In experiments designed to measure the absorption in the duodenum and the upper part of the jejunum the test solution was administered orally When the purpose was to study the absorption from more distal parts of the jejunum the test solution was administered by infusion into the proximal part of the small intestine to obtain an adequate flow through the tube The degree of absorption

was determined by measuring the ratio between the radioactivity per mg PEG of the aspirates and that of the test solution (A/T ratio). For further details about the experimental procedure see HELLSTRÖM *et al.* (1970) and BEERMANN, HELLSTRÖM & ROSÉN 1971a & b).

The plasma level of label was followed for 1-2 days and the excretion of radioactivity in the urine and the faeces for 6 days.

Material

Atropine randomly labelled with tritium was purchased from the Radiochemical Centre Amersh[illegible] England. Methylatropine, prepared from labelled atropine as described by A[illegible]
lamine the labe[illegible]
Acabel (benzilic acid ester [illegible]
phate) was obtained from Astra AB, Sweden.

When the radiopurity was below 98 per cent the label was purified by chromatography on a Sephadex column on the day prior to or on the day of the experiment. The radiopurity was ascertained by electrophoresis or paper chromatography.

Determination of radioactivity

All determinations were performed with a liquid scintillation spectrometer (Packard Tri Carb model 3003). Aliquotes (1 ml) of untreated urine and gastrointestinal aspirates were mixed with 15 ml of scintillation liquid (BRAY 1960). Samples of blood plasma were dried at 30°C. Aliquotes of the dried powder were assessed for radioactivity with a combustion method, modified from SCHÖNIGER (1955). Faeces were analyzed for radioactivity as described by HELLSTRÖM *et al.* (1970).

Electrophoretic and chromatographic analyses

High voltage electrophoresis and ascending paper chromatography were performed as described previously (BEERMANN, HELLSTRÖM & ROSÉN 1971a & b). Aliquotes of intestinal aspirates and the test solutions were applied directly on chromatographic papers. The paper strips were subsequently cut into separate segments and the radioactivity was determined by the combustion method.

Results and Discussion

The present technique of calculating the absorption of the drugs from the upper gastrointestinal tract should only be accurate under certain conditions. 1. PEG has to be non absorbable and resistent to modification. 2. The time of its passage through the ventricle and the intestine must equal that of the drug investigated. 3. The drug should not undergo disposition. 4. The label has to remain attached to the drug.

These criteria appear to be fulfilled by the following observations. Several reports have demonstrated that PEG is neither absorbed nor modified in the upper gastrointestinal tract (FORDTRAN 1966, FLOCH 1969). The major portions of the label and PEG appeared simultaneously at the various levels of the small intestine indicating equal transit time. Upon electro-

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Results and Discussion

The present technique of calculating the absorption of the drugs from the upper gastrointestinal tract should only be accurate under certain conditions 1 PEG has to be non absorbable and resistent to modification 2 The time of its passage through the ventricle and the intestine must equal that of the drug investigated 3 The drug should not undergo dis position 4 The label has to remain attached to the drug.

These criteria appear to be fulfilled by the following observations Several reports have demonstrated that PEG is neither absorbed nor modified in the upper gastrointestinal tract (FORDTRAN 1966, FLOCH 1969) The major portions of the label and PEG appeared simultaneously at the various levels of the small intestine indicating equal transit time Upon electro-

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The Gastrointestinal Absorption of Anticholinergic Drugs: Comparison between Individuals

By

Bjorn Beermann, Kjell Hellström and Anders Rosen

Key-words Atropine – parasympatholytics – intestinal absorption

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The ratio of parenteral to oral equipotent doses varies considerably among some anticholinergic drugs Whereas atropine appears to be of equal potency irrespective of the route of administration the oral dose of propantheline and methylscopolamine has to exceed the parenteral one by a factor of 10 and 100 respectively before equal anticholinergic effects are obtained (MOLLER & ROSÉN 1968)

Studies with intestinal loops in intact rats (LEVINE & PELICAN 1964) have demonstrated considerable differences in the absorption of several anticholinergic drugs To investigate whether a poor absorption might be responsible for the low efficacy of some orally administered drugs we have adopted a radiochemical technique The present report summarizes the results so far obtained

Materials and Methods

Experimental procedure Healthy male subjects 40-60 years of age were provided with a nasal double lumen tube that was allowed to pass to the desired levels of the intestine before the start of the experiment The test solution which was administered in the morning, was made up of 1) tritiated material of the drug 2) non labelled material of the drug in amounts which – with the exception of butylscopolamine – in general produced anticholinergic effects 3) the non absorbable marker polyethylene glycol (PEG, 5 g) and 4) water In experiments designed to measure the absorption in the duodenum and the upper part of the jejunum the test solution was administered orally When the purpose was to study the absorption from more distal parts of the jejunum the test solution was administered by infusion into the proximal part of the small intestine to obtain an adequate flow through the tube The degree of absorption

Table 2

The gastrointestinal absorption, plasma concentration and urinary excretion of radioactivity after administration of ^{3}H methylatropine (5 mg, 50 μci)

	Absorption 145 cm from the nose (per cent of given dose)	Maximum plasma concentration (per cent of given dose in 3000 ml)	Urinary excretion (per cent of given dose)
Healthy subjects	19–26	1.1–2.3	15–20
Patient SN	50	7.0	39

The ratio between the amount of radioactivity excreted in the urine and faeces differed between the drugs (table 1) The urinary excretion of the label was most pronounced after administration of atropine and very minute in the experiments with butylscopolamine

Our data thus demonstrated that the degree of absorption varied between atropine, methylatropine, acabel and butylscopolamine but we were unable to find significant differences between individuals receiving the same drug In agreement with these findings the plasma concentration of label was very similar among the subjects who received atropine (BEERMANN, HELLSTROM & ROSEN 1971a), and methylatropine respectively The individual differences were somewhat higher in the subjects who had been given acabel (BEERMANN, HELLSTROM & ROSÉN 1971b) The variations between individuals were small even with regard to the pattern of the urinary and faecal excretion of radioactivity except for the subjects who received acabel Similar observations concerning the urinary excretion of label after administration of ^{3}H acabel has also been reported by BECKMANN (1966).

The present experiments were undertaken under standardized conditions The drugs were dissolved in water, the receivers were healthy and had been fasting over night. A variety of conditions may influence the drug absorption In a recent experiment we studied the uptake of labelled methylatropine in a subject who was treated with nortriptyline (sensaval®, Pharmacia), hydrochlorothiazide (dichlotride®, MSD) and vitamins because of depression and moderate hypertension The uptake of label from the upper part of the small intestine, the level of label in plasma and the excretion of radioactivity in urine markedly exceeded those of the controls (table 2) Since this is the only example of great differences between individuals we have observed so far it seems likely that the patient s medication has a bearing to the findings

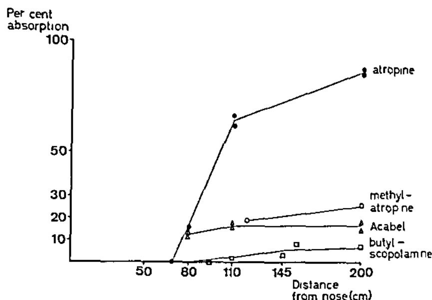


Fig 1 The absorption in the gastrointestinal tract. Each point reflects the mean of the A/T ratios recorded for one subject when the major part of PEG was in transit. ^{3}H Atropine 2 mg, 50 μci ^{3}H methylatropine 5 mg, 50 μci ^{3}H acabel 100 mg 50 μci ^{3}H butylscopolamine 50 or 100 mg, 50 μci

phoresis and chromatography the radioactivity of intestinal aspirates showed the same pattern of distribution as that of the test solution There was no evidence of a formation of tritium water in the intestine

Atropine was almost completely absorbed and particularly the duodenum and the upper jejunum were involved in this process (fig 1) The main uptake of the three quaternay ammonium compounds also occurred in the proximal part of the small intestine It amounted to 5–25 per cent of the given dose (fig 1)

The plasma concentration of the label was roughly correlated to the degree of absorption The highest levels were observed in the subjects who received atropine No radioactivity was detected in blood samples from those who were given butylscopolamine, the drug most poorly absorbed

Table 1

The urinary excretion of radioactivity in per cent of the total recovery of label in urine and faeces

Atropine	91 97
Methylatropine	18–20
Butylscopolamine	2– 3
Acabel	3–25

From the Department of Pharmacology,
Karolinska institutet Stockholm, Sweden

Variations in Gastrointestinal Functions Influencing the Absorption of Drugs

By

Sven Andersson

Abstract The passage of *various drugs through* the gastric mucosa was studied in dogs After a single intravenous injection of nortriptyline (10 mg/kg) the drug was greatly concentrated in the gastric juice The absorption of tritium labelled penicillin V from an isolated gastric pouch was increased at pH 2 compared with absorption at pH 7 *Both observations are agreement with the pH partition* hypothesis of gastrointestinal absorption of drugs By perfusion of isolated pouches of the pyloric antrum with choline it was shown that absorption of choline also was pH dependent – the absorption was decreased as the pH of the choline solution was lowered An hypothesis for the possible mechanisms *behind the pH-dependent absorption of choline is discussed. On the basis of* results from gastric secretory studies in humans the significance of gastric secretory functions for the gastric absorption of drugs is emphasized

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Very little is known about how and to what extent various disturbances in gastrointestinal functions affect drug absorption There are normally great variations in functions such as gastrointestinal secretions and motility Such variations might be even greater in diseased states and, furthermore, changes in gastrointestinal mucosal membranes may occur which could either facilitate or reduce the passage of drugs from the gastrointestinal lumen to blood Our own interest in problems concerning the passage of drugs through the gastrointestinal mucosa derives from previous work on the physiological control of gastric acid secretion

The first extensive work on mechanisms controlling the gastrointestinal absorption of drugs was carried out by BRODIE and coworkers (BRODIE 1964) In summary, their so called pH-partition hypothesis of gastrointestinal absorption of drugs is that a) most drugs are absorbed by simple diffusion in their unionized form, b) the more lipid soluble the drug, the more rapidly it is absorbed and c) weak acidic and basic organic agents show a pH-dependent absorption *It is* surprising that so little attention has been directed to the practical consequences of their theory Nobody, as

Summary

Water solutions of tritiated atropine, methylatropine, butylscopolamine and acabel together with a non-absorbable marker (PEG) were administered orally or intraintestinally to healthy subjects provided with a gastrointestinal tube The degree of absorption in the upper gastrointestinal tract differed between the drugs The uptake of atropine was efficient whereas that of butylscopolamine was very poor There were small variations in the absorption among subjects receiving the same drug No consistent evidence of a disposition of the drugs in the upper part of the small intestine was found

REFERENCES

Albanus, L , A Sundwall & B Vangbo On the metabolic disposition of methylatropine in animals and man *Acta pharmacol et toxicol* 1969, **27**, 97–111

Beckmann, R Zum Verhalten von Benzilsaure- (N, N demethyl 2 hydroxy methyl pi peridinum) – ester methylsulfat im Organismus *Arzneimittelforschung* 1966, **8A** 910–918

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Bray, G B A simple efficient liquid scintillator for counting aquors solutions in liquid scintillation counter *Ann Biochem* 1960, **1**, 279–285

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Fordtran, J S Marker perfusion techniques for measuring intestinal absorption in man *Gastroenterol* 1966, **51**, 1089–1093

Hellstrom, K , A Rosen & K Soderlund The gastrointestinal absorption and the excre tion of H^3 butylscopolamine (hyoscine butylbromide) in man *Scand J Gastro enterol*, 1970, **5**, 585–592

Levine, R R & E W Pelikan Mechanisms of drug absorption and excretion Passage of drugs out of and into the gastrointestinal tract *Ann Rev Pharmacol* 1964, **4**, 69–84

Moller, J & A Rosen Comparative studies on intramuscular and oral effective doses of some anticholinergic drugs *Acta med scand* 1968, **184** 201–209

Schoniger, W Eine mikroanalytische Schnellbestimmung von Halogen in organischen Substanzen *Mikrochimica Acta* 1955, **1**, 123–129

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major reason for this is that quantitative studies on gastric absorption of drugs in man are difficult to carry out, intragastric pH, gastric secretion and emptying rate, as well as possible reflux of intestinal content into the stomach, must be adequately controlled Such studies can be performed more easily in dogs where the above mentioned parameters can be measured Therefore, I have selected for presentation some of our experiments on the passage of drugs through the gastric mucosa in the dog

The experiment illustrated in fig 1 can be taken as a confirmation of the Brodie hypothesis The figure shows the gastric secretion of the basic drug nortriptyline (NT) during maximal stimulation of gastric acid output with betazole The dog was provided with a gastric cannula which allowed quantitative collection of gastric juice As seen from the figure, *NT was greatly concentrated in the acid gastric juice with a concentration* ratio of 25–30 between gastric juice and plasma

In preliminary studies on the absorption of ^{3}H penicillin V (^{3}H pcV) following irrigation of isolated fundic pouches in dogs we found a severalfold increase of plasma levels of ^{3}H pcV when the pH of the ^{3}H pcV-

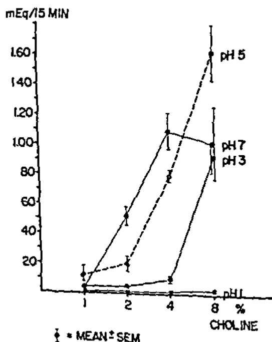


Fig. 2. Gastric acid responses to antral irrigation with choline at different pH levels in 3 Pavlov pouch dogs Each curve represents the mean ± S E M from 3–4 experiments in each dog. (From ANDERSSON & ELWIN 1968)

far as I know, has ever tried to quantitatively determine how the gastric absorption of drugs affects the pattern of their plasma concentration One

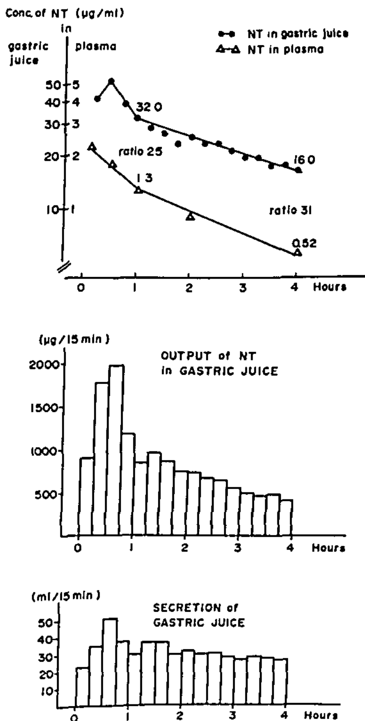


Fig 1 Excretion of nortriptyline (10 mg per kg given as a single intravenous injection) in gastric juice of dog (From SJÖQVIST et al 1969)

that fully ionized agents are poorly absorbed throughout the gastrointestinal tract. In connection with some investigations in which we studied the *stimulatory effect of various agents on the gastrin releasing mechanism of the pyloric antrum in dogs* we found choline chloride to be a potent gastrin releasing agent. Its *gastrin-releasing* action was pH-dependent as illustrated in fig 2 There is *strong evidence that the inhibitory effect of* acid on the release of gastrin *is due to reduced passage of choline through* the mucosal membranes In fact, at the highest concentrations of choline in the antral pouch, the dogs showed pronounced signs of cholinergic stimulation Furthermore, determination of choline in blood showed pronounced increase of the plasma levels of choline when the pH in the antral pouch was pH 7 whereas no elevation in plasma choline was observed with pH 1–3 Therefore, despite the fact that choline is a strong base and

Table 1

Effect of nicotinic acid on basal acid output in man.

Patient no	Mean basal acid output in meq /hour*			Peak acid response to betazole*	
	1st test (control)	0.5 g nicotinic acid 30 min before test		output meq / 30 min	conc. meq /l
		2nd test	3rd test		
1 M	1 98	2 76	2 35		
2 M	3 49	5 57	2.58		
3 M	1 22	1 96	0.27		
4 M	3 53	7 17	3 62		
5 M	0	0	–	not done	
6 M	0	0	–	achlorhydria	
7 M	0	0	–	achlorhydria	
8 M	6 08	17 46	5 15		
9 F	3 69	5.31	4 17		
10 M	2 73	3 08	3 91		
11 F	0	0	–	6 69	83
12 M	6 03	5 68	8.54		
13 M	0	0	–	7.57	94
14 M	0 90	2 99	1 11		
15 M	0	0	–	1 41	44
16 M	1 71	2 02	1 61		
17 M	0	0	–	achlorhydria	
18 F	0	0	–	1.14	29
19 F	0	0	–	1 27	36

F = female, M = male

* All values are corrected for incomplete recovery of gastric juice.

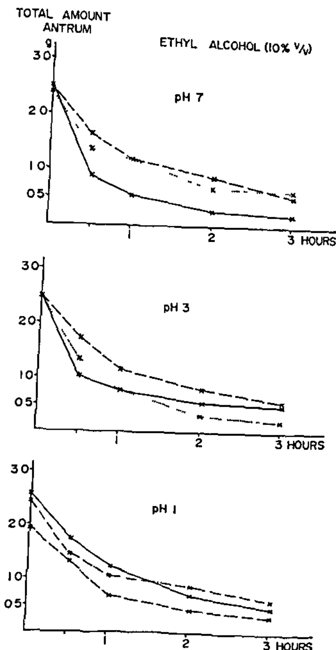


Fig 3 The disappearance of ethanol at various pH levels on instillation into isolated antral pouches in dogs (According to ELWIN & ANDERSSON, unpublished results)

solution was decreased from pH 7 to pH 2 This occurred despite the fact that the solubility of the penicillin was greatly reduced at the lower pH (ANDERSSON & NYLANDER, unpublished results) One should expect the absorption to be further increased if gastric secretion is stimulated and gastric mucosal blood flow subsequently increased

I would now like to take up another point for consideration It is taught

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Pharmacokinetics of Diazepam in Dogs, Mice and Humans

By

Eppo van der Kleijn, Jacques M van Rossum, Elly T J M Muskens
and Nico V M Rijntjes

Key words Pharmacokinetics – diazepam – distribution – metabolism

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Pharmacokinetic equations describing the course of bodyfluid concentrations have been succesfully applied for the design of dosage regimens in e g chemotherapy (KRUGER THIEMER & BUNGER 1965 & 1966, DOLUISIO & DITTERT 1969) and cardiology (JELLIFFE 1967) They are based on the assumption of a relatively simple relationship between the concentration in the bodyfluid where the drug can be assayed and the concentration in a closely related bodyregion, which is responsible for the pharmacological activity, but where it cannot be sampled In the case of chemotherapy defined criteria for activity can be used both *in vivo* and *in vitro* Since the clinical criteria for many drugs are vague and subjective, there is a great need for more objective parameters that can be used in accompaning the patients drug treatment. In many cases the kinetic parameters for the use of the regimen calculation are estimated from data after a single dose However many individual factors mediate the response and the availibility of the drugs in the bodyfluids

This paper deals with the single dose, steady state and post steady state kinetics of diazepam and its primary metabolite desmethyldiazepam Specific and general factors will be discussed that can explain the individual and species differences in pharmacokinetics

Methods

Gas chromatographic analyses in plasma of humans and dogs

Diazepam (valium® abbreviated D) and its metabolites desmethyldiazepam (DD), oxydiazepam (OD) and oxydesmethyldiazepam (or oxazepam, serax®) (O) can simultaneously and specifically be assayed in bodyfluids at the pharmacological levels using a gas chromatographic method and ^{63}Ni-electroncapture detection (MARCUCCI *et al* 1968 VAN DER KLEIJN, 1969c)

completely ionized within the pH-range used, it passed through the mucosa at higher pH-levels and absorption was almost completely blocked at low pH-levels Reduced permeability of the gastric mucosa at low pH levels cannot be explained by for example destruction of the surface epithelium by acid, since absorption of an unionized agent such as ethanol was not affected by pH-changes (fig 3) We have no ideas about the mechanism involved in the absorption of choline, but our hypothesis is that choline might be bound to and transported by some acidic carriers which are affected by the acid gastric content, possibly fatty acids in the lipid membrane Other quarternary compounds such as some anticholinergic agents show similar characteristics of gastric absorption

I am convinced that gastric absorption plays an important role in connection with oral administration of drugs which from their physico chemical properties can be expected to pass the gastric mucosal membranes In such cases the actual pH of the gastric content is of significance We know that gastric pH varies during the day and in connection with meals and, even more important, it varies greatly between individuals Table 1 exemplifies this In a group of patients – 40–65 years of age – with arterio sclerotic diseases and hyperlipoproteinaemia 50 per cent had basal achlorhyfria and, among these 9 subjects, three had true achlorhydria The same high frequency of achlorhydria in hyperlipaemic patients was also found by MARKS *et al* (1962) In their control group of individuals without arteriosclerotic and gastrointestinal diseases less than 10 per cent had basal achlorhydria and none had true achlorhydria These large interindividual differences in gastric pH-levels may explain the great variations which have been observed in, for example, peak plasma levels following oral administration of certain drugs

Acknowledgement

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REFERENCES

Andersson, S & C E Elwin Influence of acidity on release of gastrin by chemical agents In *The physiology of gastric secretion* Ed L S Semb & J Myren Universitetsforlaget, Oslo, Norway, 1968

Brodie, B B Physico-chemical factors in drug absorption In *Absorption and distribution of drugs* Ed T B Binns Edinburgh and London E & S Livingstone Ltd 1964

Marks, I N, S Bank, L H Krut & B Bronte-Stewart Gastric secretion and alimentary lipaemia in ischaemic heart disease *Lancet* 1962 **11** 1068

Sjoqvist, F, F Berglund, O Borgå, W Hammer, S Andersson & C Thorstrand The pH dependent excretion of monomethylated tricyclic antidepressants in dog and man *Clin Pharm Therap* 1969, **10**, 826

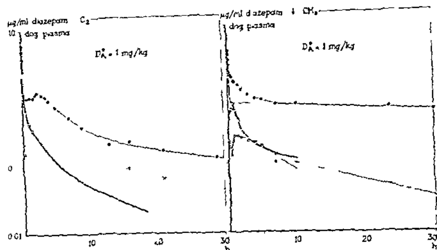


Fig 1 Concentration time course of total radioactivity in bloodplasma (• •), of total radioactivity in plasma water (•--•) of diazepam (○-○) and desmethyldiazepam (□-□) after rapid intravenous administration of 1 mg diazepam/kg in two dogs

In fig 1 it is shown that the elimination of the parent diazepam occurs along at least a triphasic curve In general the accuracy of the kinetic parameters as they can be derived from concentration-time curves after a single dose depends largely on the accuracy of the last phase of the curve (WAGNER 1969)

Unfortunately it is not possible to determine the drug long enough during this last phase It can be remarked that using the $^{14}C_2$-labeled compound a shoulder in the curve of the total radioactivity is observed This is even more pronounced for the total radioactivity in the plasma water

After the first three hours the protein binding capacity hardly changes anymore, indicating that metabolism has resulted in one or more compounds that show a protein binding capacity of about 58 %

After administration of the N-$^{14}CH_3$ labeled compound the elimination pattern of the total radioactivity is different The protein binding capacity increases during the time course

Mice

Macroautoradiography is a useful technique to study the actual significance of the plasma concentration for the distribution of the drug in the body It allows a visual localization of the drug in regions where it cannot be detected by sampling techniques Plasma may poorly reflect the kinetic processes relevant for the pharmacological activity or for distribution of drugs with selective tissue partioning or binding

The minimum level that can quantitatively be assayed is 30 ng/ml using N benzyl 5 chlor 2 aminobenzophenon, medazepam or griseofulvin as internal standard

Isotope dilution analyses in dogs

D was administered intravenously in dogs in a dose of 1 mg/kg corresponding to 2 µci/kg In one experiment diazepam labeled with ^{14}C in the C_2-position in a second experiment diazepam labeled in the N_1 CH_3-position, was used Heparinized samples of 20 ml were frequently taken at increasing intervals from a catheter placed in the femoral artery

1 0 ml plasma was used for total radioactivity measurement by liquid scintillation counting (Packard, Model 3380 AAA)

2 0 ml plasma were used for the gas chromatographic analysis of parent diazepam and metabolite (DD) 11 ml of the sample were used for the determination of plasma protein free fraction of total radioactivity

Autoradiography in mice

Distribution of the radioactive drug was studied by means of the whole body autoradiographic method of ULLBERG (1954) using a Jung K microtome (VAN DER KLEIJN 1969c) In this study diazepam–N_1–$^{14}CH_3$ was used The specific activity of the compound amounted to 25 7 mg/mci

200 µci ≈ 5 1 mg/kg was administered intravenously in the tail vein The drug was solubilized in a saline solution containing 2 % polysorbate 80

Studies in humans

The test subjects were psychiatric patients, who had been exposed to several drugs during their chronic hospitalization They were examined physically and found healthy For the sake of this study other drugs were withheld during at least one week prior to the diazepam treatment

The drug was administered as the commercially available 10 mg tablets at equal intervals of 8 hrs during 15 subsequent days After this period the drug and metabolite concentrations were followed during the next 4 days

One patient here reported received a single dose of 30 mg 24 hrs before the subchronic study, that lasted 13 days and subsequent 3 days

Samples were taken at increasing intervals after the single dose and during the post treatment period During the subchronic study samples were taken 1 hour after the intake of each dose during the first three days and 1 hour after the last dose on the subsequent days

Plasma protein binding

The plasma protein binding capacity has been determined with the preparative ultra centrifugation method as earlier reported (VAN DER KLEIJN 1969) at 70 000 rpm

Results

Dogs

The experiment in dogs are undertaken to enable a more accurate analysis of the kinetic pattern of diazepam in plasma than is technically feasible in humans

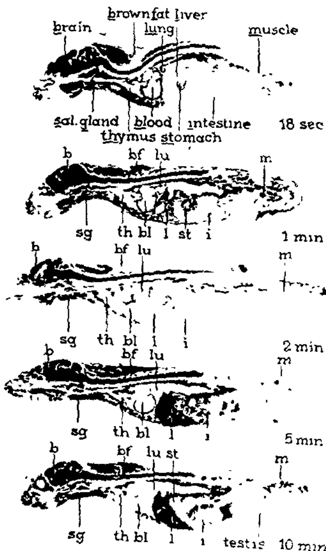


Fig. 2. Autoradiograms of diazepam-N-$^{14}CH_3$ in mice during the first 10 min, after rapid intravenous administration of 5.1 mg/kg. White areas correspond to presence of radioactivity. Notice the rapid disappearance of the radioactivity from the body as compared with earlier autoradiograms using diazepam-$^{14}C_2$ (VAN DER KLEIJN 1969).

Earlier studies (VAN DER KLEIJN 1969a) using $^{14}C_2$-labeled diazepam demonstrated that although the parent drug is rapidly eliminated, the metabolites are long retained in the body, e g in the gastrointestinal tract the liver and the white matter of the spinal cord and the brain By using the N_1-$^{14}CH_3$ labeled compound it can be confirmed that the parent drug is very rapidly eliminated No significant excretion of radioactive material into the stomach can be observed in the presented autoradiograms At short periods after injection a rapid uptake in the adipose tissue is evident The elimination from the brain proceeds faster than from the bodyfat The initial uptake in the spinal cord, the gray matter of diencephalon, and cortex is similar to other ataractic drugs No long retention of material in the white matter can be observed in these mice

A small amount of radioactive material is excreted into the gall bladder and subsequently into the intestine The drug shows an initial high affinity for the myocardium After 1 hour virtually no drug can be observed in tissues other than the excretory organs

Remarkable is the uptake in bone marrow, salivary glands lachrymal glands and the skin that can be observed even after 18 hrs following the administration (figs 2 and 3) The patterns after 4 hrs and later resemble the distribution of radioactivity following the injection of radioactive formal dehyde (HESPE & PRINS, personal communication 1969)

Studies in humans

In earlier studies (DE SILVA *et al* 1966, VAN DER KLEIJN 1969c) it was observed that after a single oral dose of diazepam it was not possible to estimate the individual kinetic parameters because of the erratic course of the concentration

After an initial rapid absorption phase and subsequent rapid decline, a recovery of the level was observed followed by a much slower elimination part This latter phase, with a half life longer than the dosage interval, is responsible for accumulation during chronic treatment under the condi tions that there are no changes in distribution volume or metabolic capacity The analytical minimum concentration that could be assayed also did not allow a conclusion regarding the metabolite after one dose

The observed rise in plasma level was correlated with food intake and will be discussed later Fig 4 gives an example of a patient where this recovery of the concentration has not been observed initially and a biphasic elimination curve can be shown after a single dose of 30 mg diazepam During the subchronic treatment of 3 doses of 10 mg daily, accumulation of both diazepam (D) and desmethyldiazepam (DD) takes place D reaching a steady state after approximately 4 days and DD reaching it after about 5 days After the treatment has been stopped both the D and the DD concen

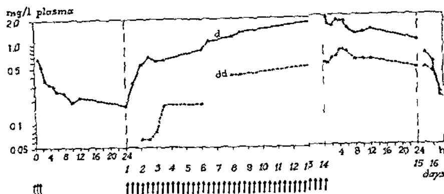


Fig 4 Blood plasma concentration time course of diazepam (D) and its metabolite desmethyldiazepam (DD) in a psychiatric patient after a single dose of 30 mg, during subchronic treatment with 3 times 10 mg daily during 13 days and during subsequent 3 days following the treatment Notice that the initial elimination pattern differs from the post treatment pattern (patient dB)

tration show an erratic time course (fig 5) The earlier shown rapid initial decay is no longer observed anymore This can theoretically be expected as will presently be discussed Within certain limitations a half-life of 24 hrs for D can be estimated in patient d B

The concentration time curves represented in fig 6 show the accumula-

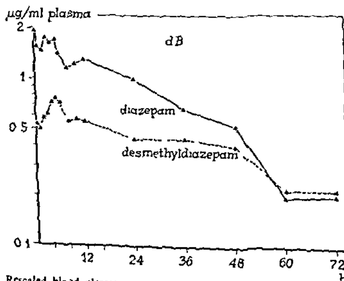


Fig. 5 Rescaled blood plasma concentration time course of D and DD during the post subchronic treatment period presented in fig. 4 Notice that during the initial decay an erratic course is shown of both D and DD

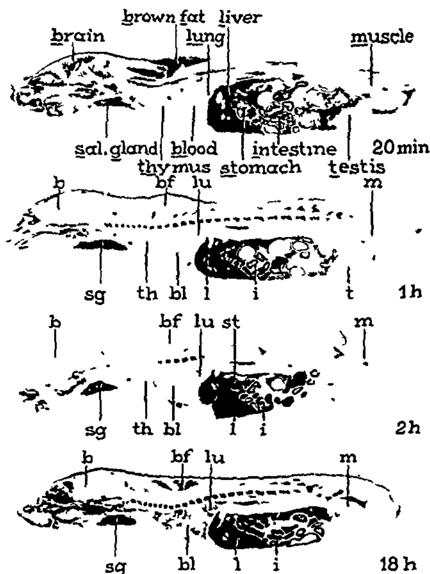


Fig 3 Autoradiograms of diazepam N $^{14}CH_3$ in mice during the period of 20 min 18 hrs after rapid intravenous administration

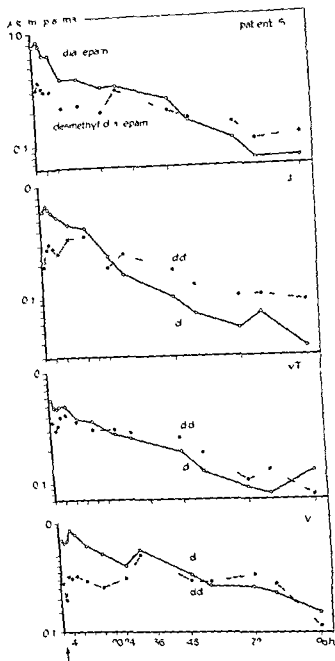


Fig. 7 Rescaled blood plasma concentration time course of D and DD during the post treatment period in the four patients represented in fig 6

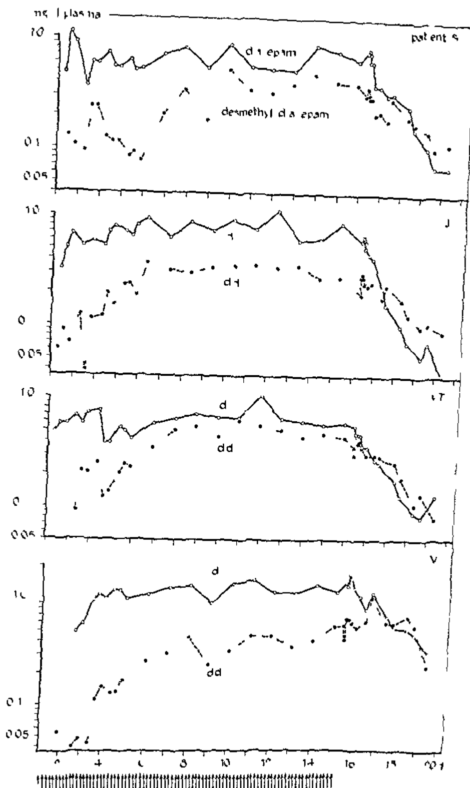
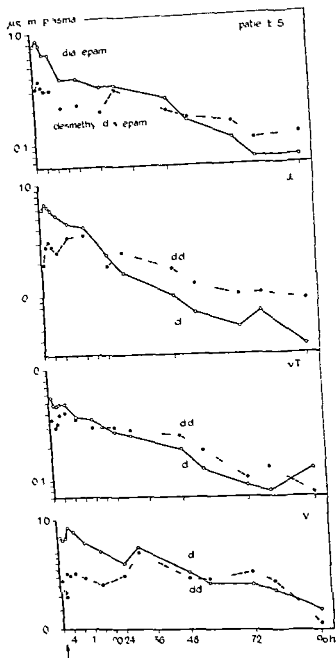


Fig 6 Plasma concentration time course of *D* and *DD* in four psychiatric patients during subchronic treatment with 3 times 10 mg D daily over 15 days and the subsequent 4 days following the treatment.



F g. 7 Rescaled blood plasma concentration time course of D and DD during the post treatment period in the four patients represented in f g 6

Table 1

$\overline{C}_{pl}$ = average blood plasma concentration at the steady state plateau level (mg/l)
Subscriptions D and DD = diazepam and desmethyldiazepam
$t\frac{1}{2}$ = biological half life (hr)
k_{12} = rate of elimination of diazepam (hr^{-1})
k_{23} = rate of elimination of desmethyldiazepam (hr^{-1})
M Clearance = metabolic clearance, the volume of the apparent steady state volume of distribution cleared per unit of time (l/hr)

Patient	Dose (mg)	Interval (hr)	$\overline{C}_{Dpl}$	$\overline{C}_{DDpl}$	$t\frac{1}{2}D$	$t\frac{1}{2}DD$*	Body weight (kg)
d B	10	8	1 50	0 40	24	48	95
S	10	8	0 80	0 43	24	56	68
J	10	8	0 85	0 35	20	42	60
v T	10	8	0 70	0 52	30	44	80
V	10	8	1 30	0 46	42	96	78 5

	k_{12}	k_{23}*	k_{23}**	$t\frac{1}{2}DD$**	M Clearance	V_D	Δ BW %
d B	28 8 × 10 3	14 1 × 10 3	108 0 × 10 3	6 4	0 833	28 9	30
S	28 8 × 10 3	12 3 × 10 3	53 6 × 10 3	12 9	1 563	54 3	77
J	34 7 × 10 3	16 5 × 10 3	84 3 × 10 3	8 2	1 471	42 4	77
v T	23 1 × 10 3	15 7 × 10 3	31 1 × 10 3	22 3	1 786	77 3	96 5
V	16 4 × 10 3	7 2 × 10 3	46 3 × 10 3	15 0	0 962	58 7	74

V = apparent volume of distribution at the steady state (l)
Δ BW = fraction of bodyweight taken by V
* This value has been determined from the tangent of the post treatment curve
** This value has been determined with the aid of eq 12

tion patterns of D and DD during subchronic treatment with 3 divided doses of 10 mg daily and the post treatment course In fig 7 the time basis is rescaled allowing a better picture of the elimination pattern The pharmacokinetic parameters that can be obtained from this post treatment curve also suffer from inaccuracy An estimate of the biological half life of the drug in humans is made in table 1 Estimation of the half-life of DD that has qualitatively a similar but quantitatively a slightly lower pharmacological activity (RANDALL *et al* 1965) has theoretical and practical implications that will be described in the discussion

Plasma protein binding of D has been determined to be virtually linear over the therapeutic concentration range (fig 8)

A protein binding capacity of 96 % has been calculated

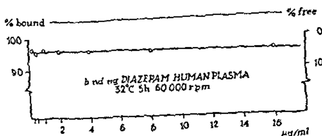


Fig. 8 Blood plasma protein binding capacity of diazepam in human plasma, at 70 000 rpm

Discussion

There are two basic questions that have to be considered for every drug and individual before one can depend on pharmacokinetic parameters calculated from plasma concentrations to predict or correct dosage regimens

First, what is the relation between the concentration in the body fluid where the drug can be assayed and the concentration in the organ, tissue or humour where the drug will demonstrate its activity? Second, what is the relevance of a peripheral or regional concentration for the nature and intensity of the action?

The first question may be answered by the use of mathematical models commonly used in pharmacokinetics The principle on which pharmacokinetics operate is that the relationship between the concentration of the drug in one body and the concentration in one or more peripheral bodyfluids can be expressed in mathematical terms In many cases an excellent fit of data has been obtained assuming one, two or three mathematical compartments and first order mass transfer To extend the meaning of these abstract compartments there is a tendency to identify them with anatomical or physiological regions and spaces This appears only to be valid in a restricted number of compounds e g iodine albumine, inuline, antipyrine etc

The value of this approach largely depends on the accuracy, the specificity of the assay, the connected detection range and the sampling protocol These factors will determine, whether or not the different compartments can be distinguished.

For many drugs the hypothetical peripheral compartments may indeed show the postulated homogeneity

However, many other drugs show selective tissue affinity, that mathematically moreover will often be of a non linear type In humans it is practically impossible to estimate a peripheral drug concentration and

much of our knowledge is based on animal experiments But here too, technical improvements have to be made in order to detect these relations between the plasma and the receptor or "molecular" level

Because of the anticipated difficulties after a single dose where distribution, elimination with linear and non-linear kinetics can complicate the patterns in the different compartments, it is often more simple to study the plasma concentration pharmacological activity relationship at the steady state level of the drug However it can be very difficult to establish the same clear multi steady state level-pharmacological response relationship as have succesfully been found in isolated organ experiments (Ariens et al 1964)

When biological half-life and dosage interval allow only negligible concentration fluctuations and when absorption is relatively rapid and complete,

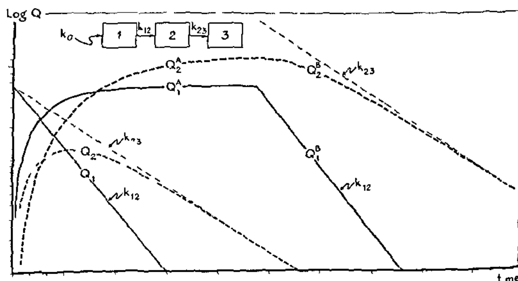


Fig 9, insertion Schematic representation of a catenary chain system with zero order infusion (k_0) into compartment 1 The rate constants representing the mass transfer between the compartments are considered to be of the first order type The drug as well as the metabolites 2 and 3 are supposed to be distributed in one compartment Fig 9 Theoretical amount time curves after a single dose of drug of its metabolite, during linear infusion and following the infusion period For the sake of a clear illustration the elimination of the drug is 2 times faster than of the metabolite, this in contrast with most of the observations with D and DD in humans. $k_0 = 0\ 45$ unit weight/time, $k_{12} = 0\ 9$ time^{-1}, $k_{23} = 0\ 45$ time^{-1} Q_1 and Q_2 are the amounts of drug and of metabolite after a single dose of $Q = 0\ 45$ unit weight. The subscriptions A and B indicate the infusion – and the post infusion period respectively When both drug and metabolite are pharmacologically active then the elimination of the active moiety is slower than of the parent compound even when the metabolite is eliminated more rapidly

then apparent linear zero order infusion kinetics can be applied to describe the accumulation of the drug and metabolites in plasma

The theoretical aspects of linear infusion have recently been reviewed by GIBALDI (1969) and in a more general form by LOO & RIEGELMAN (1970) From their work it becomes clear that since accumulation takes place it will technically be easier to decide on the kinetic characteristics of a drug. It also explains that when there are relatively large differences between the rate constants in e g a two compartment model it becomes more difficult to distinguish the two phases of the post infusion plasma curves as the time of infusion is increased

This can also explain the change in half life of tetracyclines (DOLUISIO & DITTERT 1969) during subchronic treatment where an initially unobserved slower elimination phase may control the post steady state curve This explanation is also valid for the difference in apparent elimination rate of diazepam after a single dose and after subchronic treatment (fig 4)

The concentration pattern as reported in fig 6 may be treated in a simple catenary chain system for the precursor-product relationship, according to the insertion in fig 9, assuming a one compartment model (1) for the drug (D) with zero order infusion with rate k_o (mg/hr) and one compartment (2) for the metabolite (DD)

In the case of diazepam both the parent compound D and DD are completely eliminated by metabolism since no intact material has been found in urine or faeces

The mathematical equations have been derived by RESIGNO & SEGRE (1966) and WAGNER (1969)

$$Q^1_A = (k_o/k_{12}) \quad (1 - e^{-k_{12}t})$$

$$Q^2_A = (k_o/k_{12}) \quad [1 + (k_{23}e^{k_{12}t} - k_{12}e^{k_{23}t})/(k_{12} - k_{23})] \tag{2}$$

$$Q^3_A = k_o(k_{12} - k_{23} + 1)/(k_{12} - k_{23}) \quad [(k_{12}/k_{23})e^{k_{23}t} - (k_{23}/_{12})e^{k_{12}t}] \tag{3}$$

When the linear infusion is stopped at time $t = t^1$ and the quantity of the drug if followed during time $t - t^1 = t^{11}$ then the following set of equations apply

$$Q^1_B = (k_o/k_{12}) \quad (1 - e^{k_{12}t^1}) \quad e^{k_{12}t^{11}} \tag{4}$$

$$Q^2_B = (k_o/k_{12}) \quad (1 - e^{k_{12}t^1}) \quad [k_{12}/(k_{23} - k_{12})] \quad (e^{k_{12}t^{11}} - e^{k_{23}t^{11}}) + (k_o/k_{23}) \quad [1 + 1/(k_{12} - k_{23})(k_{23}e^{k_{12}t^1} - k_{12}e^{k_{23}t^1})] \quad e^{k_{23}t^{11}} \tag{5}$$

$$Q^3_B = k_{23} \int_{t^1}^{t^{11}} Q_2^B \, dt \tag{6}$$

When the infusion time t^1 is great, then equations 4 and 5 can be simplified to

$$Q^1_B = (k_o/k_{12})e^{k_{12}t^{11}} \tag{7}$$

$$Q^2_B = (k_o/k_{23})\ e^{k_{23}t^{11}} + [k_o/(k_{-3} - k_{12})](e^{k_1 t^{11}} - e^{k_s t^{11}}) \tag{8}$$

Since the amount excreted (Q^3) has not been assayed in this study it will not be considered further

At $t^{11} = 0$

$$Q^1_B = k_o/k_{12} \tag{9}$$

and

$$Q^2_B = k_o/k_{23} \tag{10}$$

In these equations Q is quantity of the drug, the superscriptions 1, 2 and 3 refer to the compartments Subscriptions A and B refer to the infusion and post infusion period respectively, k stands for the first order rate constant (hrs^{-1}), the subscriptions indicate the direction of the mass transfer, and t is the time after starting the infusion Also

$$Q = C\,V \tag{11}$$

where C is the concentration and V is the apparent distribution volume at the steady-state

When we, with all reserves, assume the same distribution volume for Q_1 and Q_2 then k_{23} can be calculated from the ratio of the steady-state plasma concentrations, when k_{12} is determined from the post treatment curve

$$\frac{C^1}{C^2} = \frac{k_{23}}{k_{12}} = \frac{t\frac{1}{2}_D}{t\frac{1}{2}_{DD}} \tag{12}$$

The assumption of equal volumes for parent compound and metabolite is arbitrary Only studies under equal conditions with the metabolite given as parent compound can solve this problem

The post treatment or post infusion curve of the drug does not allow an accurate estimate of the rate of elimination of the metabolite The course of the curve of the metabolite (2) is determined by two terms (eq 8) The second part approaches more rapid to zero than the first part of the righthand side of the equation So theoretically only the tangent to the curve will give the k_{23} value (fig 9)

[illegible] the value of k_{23} calculated from

[illegible]

but quantitatively a slightly lower pharmacolog[illegible]

[illegible]DALL *et al* 1965, MARCUCCI *et al* 1968) If this can be translated to man then it is not the intrinsic elimination rate of DD (k_{23}) but the more complex function (eq 8) that has to be considered in the additive relation with the pharmacological activity

The catenary infusion model serves as a simplified scheme for the chronic treatment of drugs in humans A two or more compartment model for the drug may be masked by the long treatment and the erratic concentration pattern, for which individual enteral reasons are suggested When we assume that the absorption is relatively rapid and complete and that the half life of the compound is large compared to the dosage interval and thus $k_0 = Q/\Delta t$ then eq 9 can be rewritten as the equation given by WAGNER *et al* (1965) and by VAN ROSSUM AND TOMEY (1968)

$$\widetilde{C_{pl}} = 1\,44\,Q\ \ t_{\frac{1}{2}} / \Delta t\,V \qquad (13)$$

since $1/k = 1\,44\,t_{\frac{1}{2}}$

where Q is the dose, Δ t is the dosage interval and where $\widetilde{C_{pl}}$ is the average plasma plateau concentration This equation allows the calculation of the apparent distribution volume (V) of the subjects The relative large individual differences have been reported in table 1

As a consequence of the accumulation and addition of the metabolite, a higher concentration of the active substances in the brain and a longer time course of action are anticipated after subchronic treatment However, clinical observation does not reveal an extension of the activity compared with the single dose treatment Defined pharmacological criteria are necessary to allow a conclusion to this

This brings up the second question initially mentioned in this discussion about the relevance of a regional drug concentration for the pharmacological activity

Earlier experiments in mice have demonstrated that diazepam is extensively and rapidly taken up in the brain and in the bodyfat and that metabolites of this drug are also rapidly appearing in the stomach contents, the bile and subsequently in the gastrointestinal tract after intravenous injection (VAN DER KLEIJN 1969a)

It is very likely that this enterohepatic and to a smaller extend *enterogastric and enteroglandular* cycling of e g the salivary glands and lachrymal glands and subsequent sustained reabsorption contribute to the erratic and

extended concentration course of diazepam and likely other weakly basic compounds

After an initial high uptake in the gray matter of the brain the drug and metabolites are rapidly redistributed into the fiber tracts of spinal cord, brain stem and the white matter of the cortex

The gray matter of the limbic system has been recognized neurophysiologically as the site of action of diazepam and related antianxiety drugs (SCHALLEK *et al* 1964)

It is not clear yet whether the presence in the white matter has any signifi

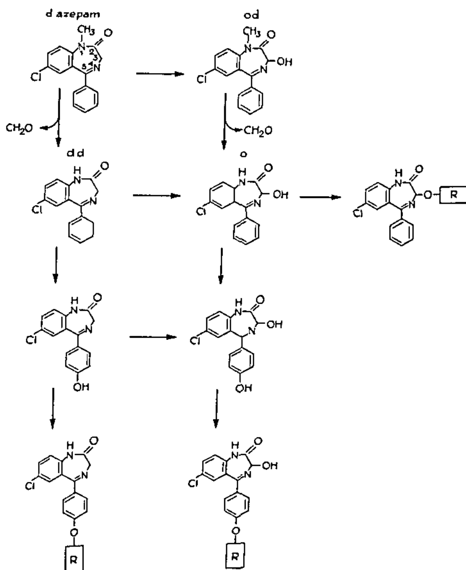


Fig 10 Schematic representation of the metabolic pathways of diazepam The abbreviations are explained under Methods

Table 2

The columns 1, 2 and 3 refer to the different phases of the concentration time curves
Biological half lives of diazepam and desmethyldiazepam in different species

Species	D			DD	$D\ ^{14}C_2$		$D\ N_1\ ^{14}CH_3$		
	1	2	3	3	2	3	1	2	3
Mouse	4½ min.	40 min.		>4 hrs		>4 hrs			
Dog	4 min	40 min	9 hrs	9 hrs	3 hrs	16 hrs	4½ min	60 min.	100 hrs
Human			24-42 hrs						

• Data from van der Kleun (1969a) derived from the heart tissue concentrations

cance for the different shades in pharmacological activity and time course of action The longest retention of the radioactivity in mice is observed in the liver, gastrointestinal tract, bone marrow, skin and salivary and lachrymal glands (fig 3)

The metabolism of diazepam has been found possibly to occur along the scheme given in fig 8 (Schwartz *et al* 1965, Schwartz *et al* 1967) Oxydiazepam and oxazepam however are only uncommonly found in human plasma

The studies in dogs and mice using radio labelled drug indicate that other metabolites that have not been assayed by the gas chromatographic method must be present (fig 1, van der Kleun 1969c) So far no qualitative and quantitative data on the chemical or pharmacological nature of these compounds in men are present

Theoretically the D → OD → O → O glucuronide pathway could be very rapid. The step from DD to O could be rate limiting and thus be responsible for the accumulation of DD in plasma A definitive answer to this problem again can only be given by studies with these metabolites given as parent compound to the same subjects

The differences and similarities in the kinetics reflecting mixing [1], distribution [2] and elimination [3] of drug and metabolite in three species are shown in table 2

The extremely slow elimination of the total radioactivity originating from $D\text{-}N_1\text{-}^{14}CH_3$ in the dog and the increasing protein binding capacity as can be concluded from the different slopes of the curves for plasma and plasma water indicates that the arisen $-\ ^{14}CH_3-$ group is taken up by the plasma proteins to a large extend

Plasma protein binding has been demonstrated to influence the pharmacokinetics of elimination for those drugs where it potentially controls the gradient over membranes (ANTON 1961) However, for the more lipophylic compounds that rapidly penetrate intracellularly the effect of the, by the binding decreased, gradient is effectively compensated (VAN DER KLEIJN 1969b)

Summary

Distribution in mice shows that diazepam is rapidly taken up in the brain, but that it is also rapidly eliminated there and slower from the body fat Retention of radioactivity can be observed in the gastrointestinal tract and the excretory organs The studies in dogs show that the drug is also rapidly metabolized resulting in identified and unidentified metabolites some of them having a longer biological half-life

Blood plasma concentrations of drug and metabolite have been followed during single dose, subchronic and post subchronic treatment with diazepam in psychiatric patients The accumulation of metabolites in the gastrointestinal tract as observed in animal studies after intravenous administration is suggested to influence the kinetics of absorption and reabsorption together with environmental factors

Acknowledgements

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REFERENCES

Anton, A H A drug induced change in the distribution and renal excretion of sulfonamides *J Pharmacol* 1961, **134**, 291–303

Ariens, E J, A M Simonis & J M van Rossum *Molecular pharmacology* Ed E J Ariens Academic Press, New York and London, 1964 **1**, 119

De Silva, J A F, B A Koechlin & G Bader *Bloodlevel distribution patterns of* diazepam and its major metabolite in man *J Pharm Sci* 1966 **55** 692–702

Doluisio, J T & L W Dittert Influence of repetitive dosing of tetracyclines on biologic half life in serum *Clin Pharmacol Therap* 1969 **10**, 690–701

Gibaldi, M Estimation of the pharmacokinetic parameters of the two compartment open model from post infusion plasma concentration data *J Pharm Sci* 1969 **58**, 1133–1135

Jelliffe R. W A mathematical analysis of digitalis kinetics in patients with normal and reduced renal function *Math Biosci* 1967, 1, 305–325

Kruger Thiemer E. & P Bunger The role of the therapeutic regimen in dosage design (part I and II) *Chemotherapia* 1965 10, 61–73

Kruger Thiemer E. & P Bunger The role of the therapeutic regimen in dosage design part I and II) *Chemotherap a* 1966 10 129–144

Loo J C K. & S Riegelman Assessment of pharmacokinetic constants from post infusion blood curves obtained after i v infusion *J Pharm Sci* 1970 59, 53–55

Marcucci F., A. Guaitani, J Kvetina, E Mussini & S Garattini Species differences in diazepam metabolism and anticonvulsant effect *Eur J Pharmacol* 1968, 4, 467–470

Marcucci F R Fanelli & E Mussini A method for gaschromatographic determination of benzodiazepines. *J Chromatogr* 1968, 37, 318–320

Randall L. O C. L. Scheckel & R. F Banziger Pharmacology of the metabolites of chlordiazepoxide and diazepam *Curr Therap Res* 1965, 7, 590–606

Resigno A & G Serge *Drug and tracer kinetics* Blaisdell, New York, N Y, 1966 29–40

Schallek, W F Zabransky & A. Kuehn Effect of benzodiazepines on central nervous system of cat. *Arch Int Pharmacodyn* 1964 149, 467–483

Schwartz, M A B A Koechlin, E Postma, S Palmer & G Krol Metabolism of diazepam in rat, dog and man *J Pharmacol* 1965, 149, 423–435

Schwartz, M A P Bommer & F M Vane Diazepam metabolites in the rat *Arch Biochem Biophys* 1967 121, 508–516

Ullberg, S Studies on the distribution and fate of S35-labeled benzylpenicillin in the body *Acta Rad ol* 1954 suppl 118, 22–31

Van der Kleijn E Kinetics of distribution and metabolism of diazepam and chlordiazepoxide in mice *Arch Int Pharmacodyn* 1969a, 178, 193–215

Van der Kleijn, E Protein binding and lipophylic nature of ataractics of the meprobamate- and diazepine group *Arch Int Pharmacodyn* 1969b 179, 225–250

Van der Kleijn E *Pharmacokinetics of ataractic drugs* Thesis University of Nijmegen, Ste Catharina Press Bruges Belgium 1969c.

Van Rossum J M & A. H. M Tomey Rate of accumulation and plateau concentration of drug after chronic medication *J Pharm Pharmacol* 1968 20, 390–392

Wagner J G J I Northam C. D Alway & O S Carpenter Bloodlevels of drug at the equilibrium state after multiple dosing *Nature* 1965, 207, 1301–1302

Wagner J G *Pharmacokinetics*. J M Richards, Publisher, Grosse Point Park, Michigan 1969

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Biliary Drug Excretion and Stimulation of Bile Flow

By

Friedrich Wilhelm Koss[1]), Helmut Pelzer[2]) and Zdravko Kopitar[2])

The initial impetus for these studies which we shall describe here came from a swedish scientist A couple of years ago Ivar Sperber from the Department of Animal Physiology in Uppsala made a new approach to the theory of bile formation He was the first who demonstrated the parallelism between excretion rates of foreign compounds, mainly dye stuffs, and the production of bile and concluded from his results that osmotic factors could be the cause of the bile stimulation

In order to get more information, particularly about quantitative relationships, an analytical method is necessary that is capable of assaying not only the original drug in bile but also all metabolites which are derived from this substance The use of spectrophotometric methods is limited to metabolites with known structure and known extinction ratios The method of choice is the radioactive labeling of the drug in a position, which is not destroyed during liver passage Table 1 contains substances labeled with ^{14}C or ^{35}S, which are used as bile flow stimulating agents Other compounds listed are known to be eliminated with the bile These substances, dissolved either in saline or in olive oil were administrated to male Wistar rats by means of a small polyethylene catheter inserted in the small bowe A second catheter was inserted in the common bile duct which was connected to the exterior through the abdominal incision

Bile was collected continuously over 1 hour periods Radioactivity was counted in the bile samples From the results the molar concentrations and the cumulative excretion of the radioactive substances were calculated (fig 1)

Highly potent choleretic agents were found in blood and other tissue to only a small extent An autoradiogram of a rat, 1 hour after administration of ^{14}C-DCH-21, a substance which is a typical potent choleretic, shows that high radioactive rates are detectable mainly in the liver and in the duodenum (fig 2)

Table 1

Labelled substances.

Substance	No.
1 ^{14}C-1 phenyl 1 hydroxy ethane	(I)
	(II)
	(III)
	(IV)
	(V)
24 ^{14}C-3α,7α 12α trihydroxycholanic acid	(VI)
1 ^{14}C-phenylacetic acid	(VII)
1 ^{14}C-phenylbutyric acid	(VIII)
1 4,5-^{35}S-3-(p-methoxyphenyl)-4,5-dithiacyclopentene-2-thion (1)	(IX)
2-phenyl-quinoline-4-^{14}C-carbonic acid	(X)
2 6 ^{14}C 2 6-bis-(diethanolamino)-4 8-dipiperidino-pyrimido-(5,4-d) pyrimidine (dipyridamol)	(XI)
phenol tetrabromopthaleinsulphonic acid	(XII)
9-^{14}C-9-(o-^{14}C-o-carboxyphenyl)-6-hydroxy 3 isoxantheone (fluorescein)	(XIII)
2-carboxymethylene-3-ethyl 5 piperidino-thiazolidone-(4)	(XIV)
2 (2 hydroxy 3n hexoxy propoxy) benzoic acid (= D-CH 21) *(sodium salt)*	(XV)

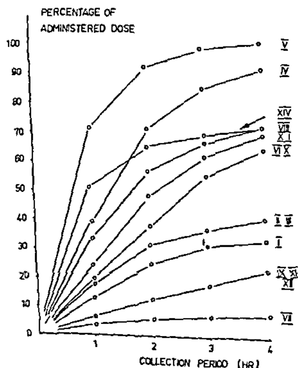


Fig 1 Cumulative excretion of radioactive substances in bile of anaesthetized rats after single intraduodenal injection of the labeled drugs



Fig. 2. Autoradiogram of a rat 1 hour after intragastral administration of ^{14}C 2-(2-hydroxy-3n-hexoxy propoxy) benzoic acid

In liver the radioactivity is located in the bile cannulas (fig. 3)

So far, only the total excretion of ^{14}C and ^{35}S respectively in the bile has been considered. It was realized that choleretic agents, which were either sparingly soluble or insoluble in water, remain in solution after excretion in the bile. Obviously, the labeled compounds are not excreted as the original drugs. Indeed, investigations into the chemical behaviour of the substances

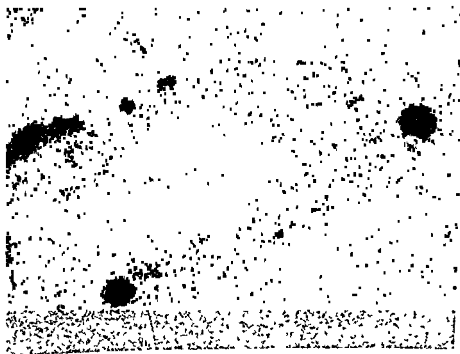


Fig. 3. Autoradiogram of a liver 6 hours after oral administration (magnification × 35)

in the bile disclosed a structure different from that of the administered drug The excretion of the original substances administered duodenally was found to be no more than 15 % In many cases the drugs were conjugated with glucuronic acid Carbinols, for instance I, II, III, IV, were eliminated in the form of their water soluble glucuronides

Substance XI was also excreted chiefly as monoglucuronide, whereas fluorescein was metabolized to mono- and diglucuronide After the administration of 2 (2 hydroxy 3n hexoxy propoxy)benzoic acid a carboxyl-glucuronide and a lactone could be isolated from bile The conjugation of cholic acid with taurine and glycine proceeds rapidly in the rat liver

Most of the ¹⁴C excreted after administration of labeled dehydrocholic acid was found in the bile in the tauro-trihydroxycholanic acid region of thin layer chromatograms Small amounts were excreted as glycine conjugates Obviously reduction of the keto group precedes conjugation of the carboxyl-group with taurine and glycine respectively Glycine is also a component of metabolites of phenylacetic acid and phenylbutyric acid

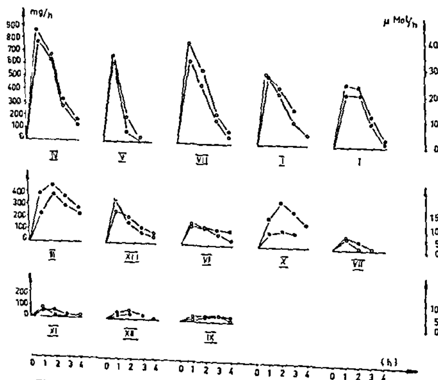


Fig. 4 Parallelism of excretion rates (µmol) and increase of bile flow (mg)

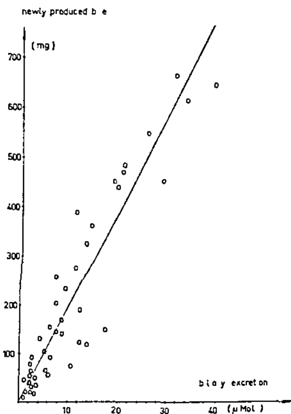


Fig 5 Straight line regression between biliary excretion (µmol) and newly produced bile (mg/hr)

Following insertion of the polyethylene tube the rate of bile flow remained fairly constant in control animals as long as the cannula remained in position The normal bile flow was about 0 5 ml/hr On the other hand, the compounds investigated caused an increase in bile flow immediately after their administration

Some active choleretics doubled or tripled the bile rate, whereas other compounds had very little effect

Fig 4 shows the extra production of bile in the hours following administration of the substances

These values are calculated by subtracting the average bile flow of the control period from the bile volume produced after drug administration

In these diagrams the molar concentrations of the choleretic substances in the bile are also shown The parallelism of the elimination curve and the bile flow is striking In both curves the maxima are reached between the first and the second hour A normalization of the bile flow was regained after about 4 hours In order to evaluate the correlation between bile flow and excretion a straight line regression was assumed The least square method led

to the function Y = 19.2 x Y is the bile flow, x the micromolar concentration of the choleretic agent That equation states that the excretion of one µmol of a foreign substance with the bile will give rise to flow of an additional ~20 mg. This ratio is independent both of the structure of the choleretic compound and of its metabolic fate

The better a substance is eliminated with the bile the higher is the rise in bile flow Substances excreted quantitatively with bile will be more potent choleretic agents than compounds with low excretion rate and incomplete elimination The rate of elimination can be limited by the absorption from the intestine, metabolism in the liver, and competitive removal of the original drug or its metabolites with blood of the hepatic vein

The high concentrations of the compounds investigated in bile leaves little doubt that they have to enter the biliary tract against very substantial chemical gradients It seems very likely that the alteration of the chemical structure in the liver cell, which is common to all compounds investigated leads to alteration of their permeability through cell membranes The A → B process may be the reason for the uni directional transfer of substances from blood to bile It accumulates metabolites at the bile side, if the size and polarity of the molecule inhibits rediffusion

The biochemical evaluation of events in the liver cell, and the search for possible parameters that might be correlated with the bile flow data, was started by SPERBER (1957, 1959 & 1962) From his experiments on certain dye stuffs as well as bile acids, phlorrhizin and polyethyleneglycol, he concluded that the mechanism of bile production is possibly identical with a disturbance of the osmotic equilibrium between bile and plasma by substances excreted into the bile.

We are pleased to be able to say that these ideas find a measure of support in our results

REFERENCES

Sperber I The biliary excretion and choleretic effect of some phenolsulphone pthaleins. *Acta Physiol Scand* 1957 42 Suppl 145, 129–130

Sperber I Secretion of organic anions in the formation of urine and bile *Pharmacol Rev* 1959 11 109–134

Sperber I Biliary excretion and choleresis. *Biochem Pharmacol* 1962, 8, 53

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Interindividual Differences in the Protein Binding of Sulfonamides: The Effect of Disease and Drugs[1] [2]

By

Aron H. Anton[3] and W. Theodore Corey[4]

Abstract The variation in the plasma protein binding of sulfonamides in healthy adults was minimal Additional evidence for consistency in binding came from a literature survey in which it was found that the plasma protein binding of several sulfonamides in man and laboratory animals was remarkably similar as reported by investigators from different countries Some hospitalized patients, however, do show a binding deficiency which may be due to an interaction between drugs, disease and altered plasma proteins Several drugs were found to compete with the sulfonamide for the same binding sites on the protein (albumin) molecule The plasma protein binding of a sulfonamide in six anephric patients was studied before and after haemodialysis As expected all showed a deficiency in binding Unexpectedly, however, the binding was less in the post dialysis plasma sample in three patients In some patients there appeared to be a qualitative and a quantitative change in the binding sites on the protein More definitive studies are needed with plasma protein fractions from healthy and ill subjects in the presence and absence of drugs This may lead to a better understanding of the side effects to some drugs and could result in the development of useful displacing agents

Key words Protein binding – sulfonamide binding – drug binding – binding

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The phenomenon of the plasma protein binding of drugs has been recognized for half a century, but its pharmacological significance for the patient has been appreciated only in the last two decades (GOLDSTEIN 1949, BRODIE

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[1]) These studies were conducted at the College of Medicine, University of Florida, Gainesville, Florida

[2]) Supported, in part, by National Heart Institute Grant HE-05251

[3]) Present address of Dr Anton and to whom reprint requests should be addressed Case Western Reserve University, Department of Anesthesiology, School of Medicine, Cleveland, Ohio 44106, U S A

[4]) The study with anephric patients was completed by W T C, class of 1971, University of Florida during a course in Experimental Medicine in the College of Medicine

1965) The introduction of the highly bound sulfonamides and semisynthetic penicillins in the 1950 s and 1960 s, respectively, stimulated an intense world-wide interest in this field *In vitro* it has been demonstrated unequivocally that the protein bound fraction of a variety of chemotherapeutic agents is devoid of antibacterial activity but the therapeutic significance of this phenomenon is at present unclear (ANTON 1960, DAVIS 1943, KLEIN & FINLAND 1963, KRÜGER-THIEMER *et al* 1965, KUNIN 1966, NEWBOULD & KILPATRICK 1960 ROBERTS *et al* 1961, ROLINSON & SUTHERLAND 1965, SCHOLTAN 1961, WARREN 1965/66) No doubt this uncertainty is due, in part, to other characteristics of the drug itself, e g pKa, lipid solubility, that influence its [illegible] which in turn

In this communication individual variability, drugs and disease will be examined as additional subtleties that may complicate the extrapolation of results *in vitro* to the clinical situation Results from an initial study of some of these factors have recently appeared (ANTON 1968)

Methods

Binding studies were carried out *in vitro* as previously described by equilibrium dialysis using various sulfonamides with plasma from fresh bank blood, healthy volunteers or from randomly selected hospitalized patients on a medical ward (ANTON 1960) In the study with the anephric patients the pre haemodialysis heparinized blood samples were collected just prior to the start of dialysis. Post dialysis (8 hours) samples were drawn by needle puncture of the cellophane dialysis coil five minutes before termination of the procedure The patients had been nephrectomized for advanced chronic renal disease of diverse etiologies. At the time of this study they had been maintained by haemodialysis from 11 to 30 months mean length of time since nephrectomy was 17.5 months No patient receiving a transfusion less than two weeks, or drugs 1 day prior to collection of sample was included in the study

The BRATTON & MARSHALL (1939) method for diazotizable amine was used to measure the sulfonamide in the samples (done in duplicate) after the protein first had been removed after precipitation with trichloroacetic acid Results are the average from at least two experiments which did not differ by more than 10 % Serum albumin, total protein serum protein electrophoresis, plasma electrolytes and blood urea nitrogen were done routinely on the anephric patients by the clinical chemistry laboratories (Dr John Savory) of the College of Medicine of the University of Florida

Kinetic analyses of certain data were carried out according to the Lineweaver Burk double reciprocal plot technique using the following equation (GOLDSTEIN 1949, KLOTZ *et al* 1946)

$$\frac{1}{R} = \frac{K}{N} \quad \frac{1}{(A)} + \frac{1}{N}$$

where

$$R = \frac{\text{Moles of bound drug}}{\text{Moles of protein (albumin, mol. wt. 70000)}}$$

(A) = Molar concentration of unbound drug

N = Maximum number of binding sites
K = Dissociation constant of the pretein drug complex
k = Association constant of the protein drug complex and is the inverse of K

plot

$$\frac{1}{R} \text{ vs } \frac{1}{(A)}$$

$$\text{y intercept} = \frac{1}{N}$$

$$\text{slope} = \frac{K}{N}$$

When drugs were tested for displacing activity the following equation was used to determine whether competitive kinetics applied

$$\frac{1}{R} = \frac{K}{N} \left((1 + \frac{(I)}{K_I}\right) \quad \frac{1}{(A)} + \frac{1}{N}$$

where

(I) = Molar concentration of the displacing agent
K_I = Dissociation constant of the protein displacing agent complex

It should be noted that we are dealing with a mixture of proteins when using plasma in the binding experiments However, since albumin is the major binding protein in plasma for the sulfomides, we will refer to interactions with albumin even though our studies were done with plasma

Results

Effect of temperature and albumin on binding equilibrium We carry out th binding studies overnight (16 hours) with gentle agitation at room tempera ture The data in table 1 indicate that under these conditions equilibrium i reached within 4 hours with a highly bound sulfonamide (sulfaethylthiadia zole) The rate to equilibrium is temperature dependent but the degree o binding was essentially the same at the three temperatures tested The reac tion is completed sooner with a less highly bound sulfonamide (sulfadiazine or in the absence of protein because less drug is required in the dialysis ba for the end point and not because of a difference in rate The presense o protein in the dialysis bag does not alter the rate to equilibrium since bindin is instantaneous and the limiting factor is the diffusion of the drug throug the dialysis membrane

The effect of plasma protein concentration on binding According to th law of mass action the binding of a sulfonamide is directly related to th protein (albumin) concentration This relationship is demonstrated in fig 1 for 3 sulfonamides of diverse binding propensity The graph suggests that except for highly bound sulfonamides (at least 90 % at therapeutic concen-

Table 1.

Effect of temperature on rate to binding equilibrium[1]

Minutes	5° [3]	23° [3]	37° [3] plasma	37° [3] (buffer) [4]	37° SAD [5]
	Percent of equilibrium[2]				
15	12 %	20 %	28 %	(50 %)	58 %
30	32	36	45	(70)	70
60	44	58	76	(93)	93
120	65	78	100		
240	77	93			
480	100				

1 A pooled sample of human plasma was used in the dialysis bags
2 Equilibrium was established when no further change in sulfonamide concentration within the dialysis bag occurred after an additional 24 hours of dialysis.
3 Sulfaethylthiadiazole was used in these samples
4 Phosphate buffer instead of plasma was used in these samples
5 Sulfadiazine (SAD) which is much less bound (33 %) than the sulfaethylthiadiazole (95 % at 100 μg/ml) was used in these samples.

trations) a considerable reduction (1 5 or greater dilution) in plasma protein (albumin) concentration would be necessary before a significant (two-fold) increase in unbound sulfonamide occurred The lowest albumin concentration

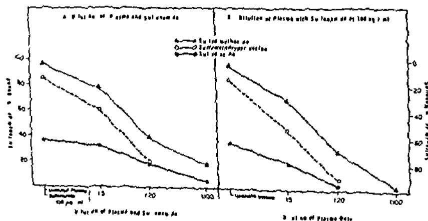


Fig. 1 The effect of dilution on the plasma protein binding of sulfonamides. A) the sulfonamide was first dissolved in the plasma and then the resultant solution was diluted so that the ratio between the sulfonamide and plasma protein remained constant. B) the binding of the sulfonamide at 100 μg/ml was determined by adding it after the plasma had been diluted. Dilutions were made with isotonic phosphate buffer, pH 7 4 (After ANTON 1968)

Table 2

Variation in protein binding of sulfonamides at 100 μg/ml in human plasma.

Sulfonamide	% bound	% unbound
Sulfadiazine, 20[1]	*29–36 (1 2)*[2]	*64–71 (1 1)*[2]
Sulfamethoxypyridazine 10[1]	80–86 (1 1)	14–20 (1 4)
Sulfadimethoxine, 10[1]	89–97 (1 1)	3–11 (3 7)

[1] Number of plasma samples
[2] Figures in branchets *indicate* the ratios between the highest and lowest values making up the range

we encountered in this study was 2 6 % (equivalent to about a 1 2 dilution) in an anephric patient Note that as predicted by the law of mass action the decrease in binding is similar when the plasma and sulfonamide are diluted (fig 1A) so as to maintain the ratio between them constant or when only the

Table 3

A Plasma protein binding of sulfadiazine in various animals

Species	Investigator (country)[1] ANTON (U S A) % bound at 100 μg/ml	Investigator (country)[1] SCHOLTAN (Germany) % bound at 100 μg/ml
Man	*36*	42
Bovine	24	*22*
Dog	17	*15*
Mouse	7	*5*
Rabbit	55	28
Rat	*45*	27

B Plasma protein binding of sulfonamides in man

Sulfonamide	Investigator (country)[1] *ANTON* (U S A) % bound at 100 μg/ml	Investigator (country)[1] SCHOLTAN (Germany) % bound at 100 μg/ml	Investigator (country)[1] NEWBOULD (England) % bound at 100 μg/ml
Sulfadiazine	*36*	*42*	*45*
Sulfamethoxypyridazine	83	85	*85*
Sulfadimethoxine	97	97	

[1] References ANTON (1960), SCHOLTAN (1961), NEWBOULD & KILPATRICK (1960) Binding was determined by equilibrium dialysis by the three *investigators*

protein is diluted (fig 1B) while keeping the sulfonamide concentration constant

Interindividual variability Contrary to the reported variability in the binding of sulfonamides among healthy individuals (SCHOLTAN 1961), we have been impressed with the consistency in this parameter This consistency is shown for 3 sulfonamides in table 2, and is based on data obtained during the past 10 years There is less than a 50 % range in the bound fraction for any of the sulfonamides Consideration of the unbound fraction, however, reveals that in the case of highly bound sulfonamides such as sulfadimethoxine a small difference in binding can produce inordinately marked changes in the unbound fraction

Consistency among investigators Additional evidence for the consistency of the protein binding of sulfonamides among healthy individuals is shown in tables 3A and 3B With the exception of the rabbit and rat (table 3A) there is remarkably good agreement on the binding of several sulfonamides in a variety of species as reported by investigators from different countries.

Variability among patients Unlike the situation in normals, marked deficiency in binding has been found in hospitalized patients The binding data from 16 patients randomly sampled on a medical ward are tabulated in table 4 The plasma protein binding of sulfadimethoxine in 12 of the patients

Table 4

Binding data on patients taking drugs *
Sulfadimethoxine (SDM)

Patient (sex age)	Diagnosis	Drugs	% SDM bound (100 µg/ml)	Binding constant (×10⁴M)	Binding sites	Albumin %
MSB (F,51)	Arthritis cancer	2	86	0 8	1 8	3 7
BP (M,59)	Lung cancer	4	92	2 1	1.5	3 0
SEB (F 44)	Hepatitis	7	87	1 6	1 6	3.0
DNJ (F 40)	Nephrosis, diabetes	14	61	0.2	2	3 2
12 Others	Hypertension cirrhosis glomerulonephritis cancer hepatitis etc.	2–8	95±1 7	1 7	2	3.9
10 Controls	Healthy	0	97±1.3	2.2	1 8	4 7

* Part of these data appeared elsewhere (ANTON 1968)

Table 5.

Effect of drugs and dilution on binding of sulfonamides to human plasma.*

Drug (μg/ml)	Sulfadimethoxine[1]		Sulfamethoxypyridazine[1]	
	% bound	% unbound	% bound	% unbound
A *Undiluted plasma*				
Control	97	3	84	16
Probenecid, 100	97	3	81	19
Probenicid, 300	93	7	72	28
Sodium salicylate, 100	96	4	72	28
Sodium salicylate, 300	86	14	54	46
Tolbutamide, 100	*94*	*6*	*70*	30
Tolbutamide, 300	83	17	45	55
P + S + T, each[2]	89	11	52	48
B *Diluted plasma (1 2)*				
Control	95	5	70	30
Probenecid, 100	88	12	64	36
Probenecid, 300	82	18	48	52
Sodium salicylate, 100	81	19	54	46
Sodium salicylate, 300	67	33	34	66
Tolbutamide, 100	79	21	48	52
Tolbutamide, 300	64	36	24	76
P + S + T, 100 each[2]	69	31	41	59

1 The percentage of sulfonamide bound was determined at 100 μg per milliliter
2 P = probenecid, S = sodium salicylate, and T = tolbutamide
* (After ANTON 1968)

was similar to the controls whereas it was decidedly less in 4 (25 %) others Note that the unbound fraction of sulfadimethoxine in these 4 patients ranged from 3 to 13 times that seen in the normal population

The remainder of this paper is concerned with our attempts to determine the basis for this binding deficiency

The effect of albumin concentration There is a direct relationship between the magnitude of binding and protein (albumin) concentration (fig 1) (other studies with albumin and globulin fractions indicate that it is mainly the albumin component to which the sulfonamide is bound in plasma) The data in table 4, however, do not show a proportionate relationship among these patients except that the control population with the highest sulfonamide binding did have the highest concentration of plasma albumin

The effect of drugs Certain drugs have been shown to interfere with the

plasma protein binding of sulfonamides (ANTON 1961), but there did not appear to be a relationship between the number or type of drugs taken by these patients and their plasma binding of sulfadimethoxine (table *4*) *Thus*, binding was most abnormal in D J N who was receiving the most drugs, but some patients in the group of 12 were taking up to 8 drugs and their binding not only was in the normal range but also was higher than M S B who was taking only two drugs As to the type of drug, patient M S B was on high doses of salicylate, a sulfonamide displacing agent, but among the group of 12 was a patient with juvenile rheumatoid arthritis who also was on large doses of salicylate and his binding was in the normal range Patient D J N was not receiving any drugs from the classes that we had found to displace sulfonamides from binding to albumin (ANTON 1961)

The displacing effect of drugs (probenecid, sodium salicylate and tolbutamide) on the binding of sulfonamides to human plasma is demonstrated in table 5 The amounts used are in the upper range of the plasma levels en-

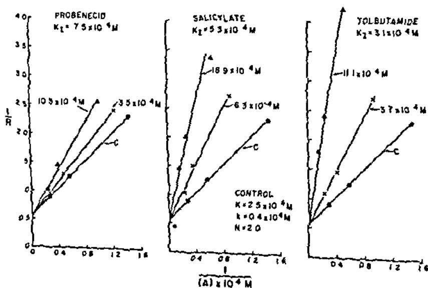


Fig. 2. Effect of drugs on the binding of sulfamethoxypyridazine in human plasma. Double reciprocal plots (see under Methods for equations) *suggest competitive inhibition between the drugs* and sulfonamide for binding sites on the protein (albumin) Tolbutamide with the lowest K_I was the most active of the three drugs tested C) control plot of sulfonamide *in* the absence of other drugs.

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Effect of drugs and dilution on binding of sulfonamides to human plasma.*

Drug (µg/ml)	Sulfadimethoxine[1]		Sulfamethoxypyridazine[1]	
	% bound	% unbound	% bound	% unbound
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Probenecid, 100	97	3	81	19
Probenicid, 300	93	7	72	28
Sodium salicylate, 100	96	4	72	28
Sodium salicylate, 300	86	14	54	46
Tolbutamide, 100	94	6	70	30
Tolbutamide, 300	83	17	45	55
P + S + T, each[2]	89	11	52	48
B *Diluted plasma (1 2)*				
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EFFECT OF DRUGS ON BINDING OF SULFAMETHOXYPYRIDAZINE IN HUMAN PLASMA

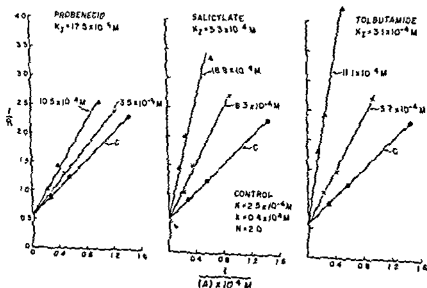


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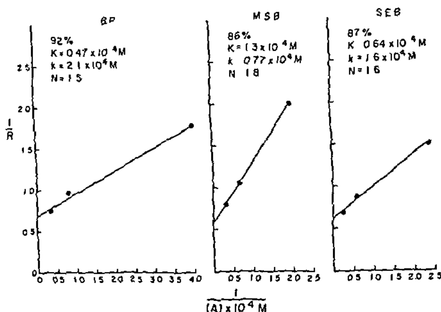


Fig 4 Double reciprocal plots for the three remaining patients in table 4 with deficient binding

Table 6

Clinical chemistry on anephric patients

Measurement	Pre Dialysis	Post Dialysis
Body weight (kg)	57 3	54 9
Haematocrit (46 %)[1]	19 8	19 8
Blood urea nitrogen (13 *mg %*)	90.3	30 2
Sodium (140 mEq/l)	*138 5*	139.2
Potassium (4 2 mEq/l)	6.0	4.3
Protein electrophoresis Mild to marked hypoalbuminaemia, some depression of serum proteins, no diagnostic pathological pattern.		

[1] Normal values in brackets

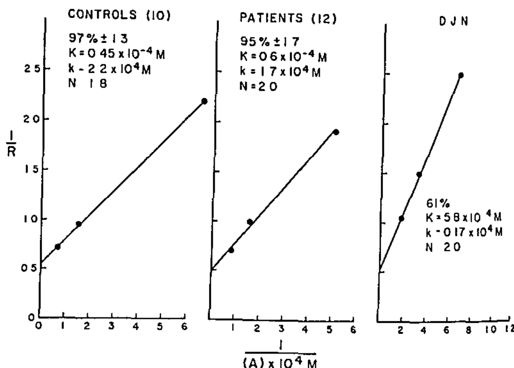


Fig 3 Double reciprocal plots of sulfonamide binding in plasma of hospitalized patients listed in table 4 Note that the affinity constant (k) for D J N is less than 1/10th that of the control group

countered clinically, and the following observations were made (1) drugs that have little displacing activity when used alone can significantly interfere with the protein binding of another drug when present in combination at that concentration, e g , probenecid, sodium salicylate and tolbutamide individually at 100 μg/ml showed little activity against sulfadimethoxine, whereas in combination they induced a fourfold increase in the unbound fraction, (2) increasing the dose of a drug may convert it from an inactive to an effective displacing agent, e g , sodium salicylate at 100 μg/ml was essentially inactive, but at 300 μg/ml it resulted in nearly 5 times as much unbound sulfadimethoxine, (3) an inactive drug may show displacing activity in the presence of a decreased plasma albumin concentration, e g , sodium salicylate at 100 μg/ml was inactive in undiluted plasma, but it caused a fourfold increase in the unbound fraction of sulfadimethoxine in diluted plasma, (4) a drug which has little activity against a very highly bound drug may displace a significant amount of a less bound agent (compare results of sulfamethoxypyridazine with sulfadimethoxine)

The interaction between the displacing agents and the sulfonamides for the

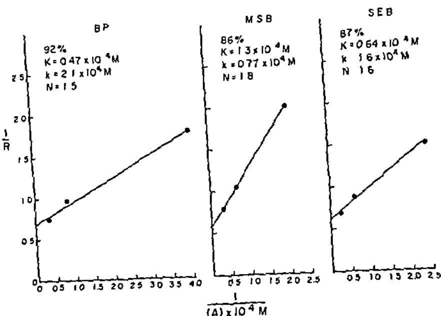


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Table 6

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Measurement	Pre Dialysis	Post Dialysis
Body weight (kg)	57.3	54.9
Haematocrit (46 %)[1]	19.8	19.8
Blood urea nitrogen (13 mg %)	90.3	30.2
Sodium (140 mEq/l)	138.5	139.2
Potassium (4.2 mEq/l)	6.0	4.3

Protein electrophoresis
Mild to marked hypoalbuminaemia some depression of serum proteins, no diagnostic pathological pattern.

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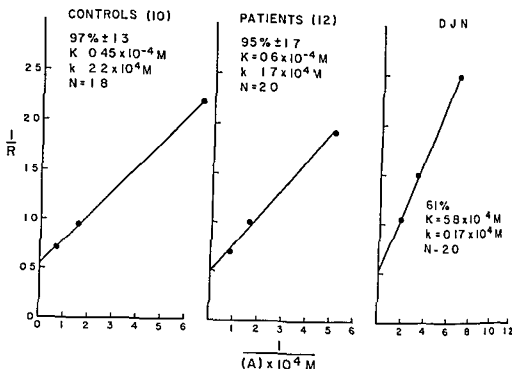


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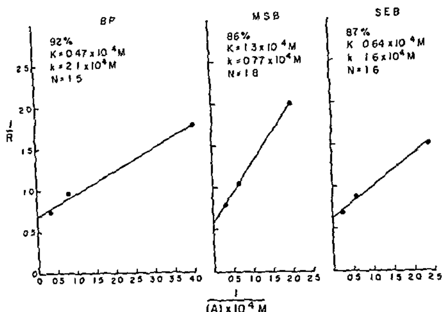


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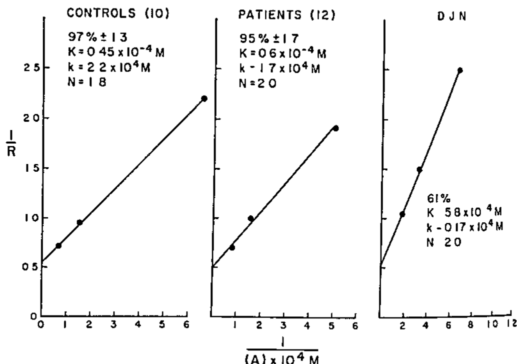


Fig 3 Double reciprocal plots of sulfonamide binding in plasma of hospitalized patients listed in table 4 Note that the affinity constant (k) for D J N is less than 1/10th that of the control group

countered clinically, and the following observations were made (1) drugs that have little displacing activity when used alone can significantly interfere with the protein binding of another drug when present in combination at that concentration, e g, probenecid, sodium salicylate and tolbutamide individually at 100 µg/ml showed little activity against sulfadimethoxine, whereas in combination they induced a fourfold increase in the unbound fraction, (2) increasing the dose of a drug may convert it from an inactive to an effective displacing agent, e g, sodium salicylate at 100 µg/ml was essentially inactive, but at 300 µg/ml it resulted in nearly 5 times as much unbound sulfadimethoxine, (3) an inactive drug may show displacing activity in the presence of a decreased plasma albumin concentration, e g, sodium salicylate at 100 µg/ml was inactive in undiluted plasma, but it caused a fourfold increase in the unbound fraction of sulfadimethoxine in diluted plasma, (4) a drug which has little activity against a very highly bound drug may displace a significant amount of a less bound agent (compare results of sulfamethoxypyridazine with sulfadimethoxine)

The interaction between the displacing agents and the sulfonamides for the

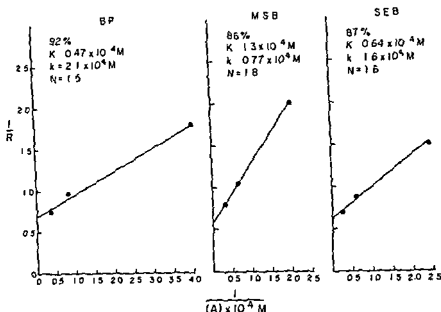


Fig 4 Double reciprocal plots for the three remaining patients in table 4 with deficient binding

Table 6

Clinical chemistry on anephric patients

Measurement	Pre Dialysis	Post Dialysis
Body weight (kg)	57.3	54.9
Haematocrit (46 %)[1]	19.8	19.8
Blood urea nitrogen (13 mg %)	*90.3*	*30.2*
Sodium (140 mEq/l)	138.5	139.2
Potassium (4.2 mEq/l)	6.0	4.3

Protein electrophoresis,
Mild to marked hypoalbuminaemia some depression of serum proteins no diagnostic pathological pattern.

[1] Normal values in brackets.

HUMAN PLASMA BINDING OF SULFADIMETHOXINE

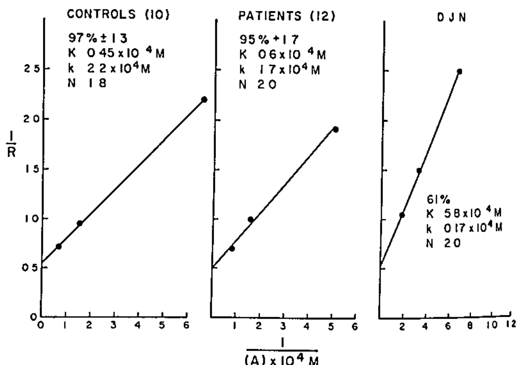


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The interaction between the displacing agents and the sulfonamides for the

Kinetic analysis of the binding in hospitalized patients The binding results from the patients in table 4 were also analyzed by the double reciprocal plot technique to determine whether the deficient binding was due to a change in affinity of the plasma protein (mainly albumin) for the sulfonamide or a change in the number of binding sites on the albumin molecule The results in fig 3 demonstrate that for the group of 12 patients whose plasma protein binding was within the normal range, the affinity of the albumin for the sul fonamide (binding constant) and the number of binding sites on the protein (albumin) molecule were similar to the controls For the other 4 patients (fig 3 and 4) it seems that the deficient binding is due to a reduced affinity of the albumin for the sulfonamide, this is particularly true for patient D J N (fig 3) whose albumin apparently had the same number of binding sites even though her plasma protein binding was the most abnormal The plots from patients B P and S E B suggest that altered binding sites may account, in part, for their binding deficiency

The effect of disease The above results suggested that the altered binding in 4 out of the 16 hospitalized patients tested could be due, in part, to drugs as well as the underlying disease In an attempt to determine the in

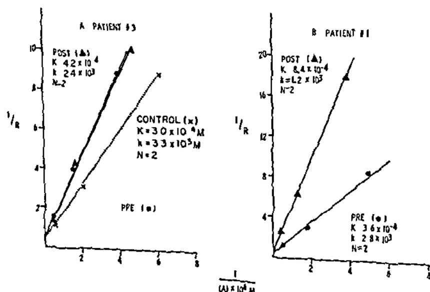


Fig. 6. Double reciprocal plots of data from patients no 1 and no 3 compared to the controls.

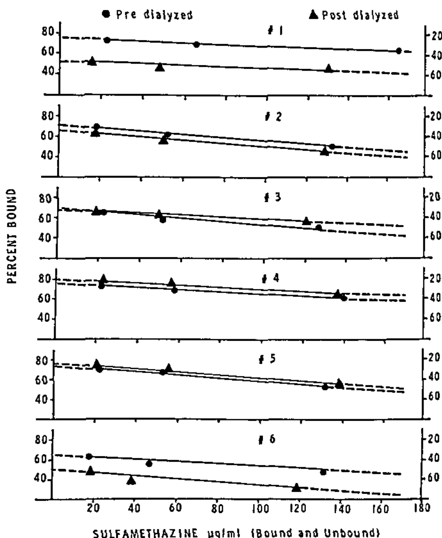


Fig 5 Binding of sulfamethazine in pre and post dialyzed human plasma from six anephric patients. Note that the binding was less after dialysis in 3 patients The plasma protein binding of the sulfonamide in all patients was less than in a control group of 3 (not shown)

binding sites on the protein (albumin) molecule was subjected to an analysis by the Lineweaver-Burk double reciprocal plot technique (see under Methods for equations) to determine whether competitive or non-competitive kinetics were involved The graphs in fig 2 suggest that the displacing agents and the sulfonamide compete for the same sites on the albumin molecule and that tolbutamide is the most active of the 3 drugs tested

at 100 μg/ml) in all the patients, but unexpectedly, the binding was decreased after dialysis in half of the patients Double reciprocal plots of the results from two of the patients were then compared to the healthy controls (fig. 6) The data from these two patients were selected because in one (patient no 1) the pre- and post-dialysis binding results were dissimilar, whereas in the other (patient no 3) they were almost identical The binding constants for both patients were less than in the controls but the number of binding sites was the same, suggesting that the altered binding was due to a decreased affinity of the protein (albumin) for the sulfonamide

The binding data for the patients and controls are compared in table 7 Although the plasma albumin and protein concentrations were decreased in all the patients, there was no proportionate relationship between this parameter and sulfonamide binding For example, patient M W had the lowest albumin concentration but her plasma binding of sulfamethazine was higher than in two other patients Note that in spite of a lowered albumin concentration and an apparent decrease in binding sites the pre-dialysis samples from patient M W had a higher binding constant than the control, suggesting a possible change in protein structure

Discussion

An indication of the clinical importance of the plasma protein binding of drugs comes from several reports attributing the exaggerated effects of certain drugs and endogenous hormones as being due to their inadvertent displacement from binding to plasma protein by the administration of another agent (BRODIE 1965, DESGREZ & DE TRAVERSE 1966) Another consequence of protein binding is the interference with the inhibitory action of a drug as well as its metabolism (ANTON & BOYLE 1964, NEWBOULD & KILPATRICK 1960) In the case of various chemotherapeutic agents it has now been established that the bound fraction is devoid of antibacterial activity (ANTON 1960, NEWBOULD & KILPATRICK 1960, KRUGER-THIEMER et al 1965, KUNIN 1966, ROBERTS et al 1961, ROLINSON & SUTHERLAND 1965 SCHOLTAN 1961) Determination in vitro of the antibacterial activity of plasma from patients receiving chemotherapeutic agents indicated that a sufficient amount of the unbound fraction was present to exert an anti bacterial effect (KRUGER-THIEMER et al 1965, ROLINSON & SUTHERLAND 1965, SCHOLTAN 1961) SCHOLTAN (1961) found some correlation between the unbound fraction of several sulfonamides and their curative effects against bacterial infections in mice MADSEN et al (1963) and BATTERMAN et al (1966) purported to show that the high plasma protein

fluence of disease alone on binding we carried out a study in anephric patients This population was selected because its members are similar with respect to pathology and treatment, and they would be available on a long term basis Also, blood chemistries including serum protein electrophoresis on these patients were available from clinical chemistry Furthermore testing the plasma sulfonamide binding capacity of these patients before and after dialysis should provide an indication as to whether observed changes were due to circulating endogenous metabolites since such substances presumably would be reduced in concentration after dialysis

The clinical chemistries on the six patients studied are summarized in table 6 The two striking abnormalities are the high blood urea nitrogen and the low haematocrit Serum protein electrophoresis did not reveal any particular pattern that could be associated with the altered binding except for the hypoalbuminaemia The decrease in blood urea nitrogen after dialysis implies that the levels of other abnormally elevated circulating substances also were reduced by the dialysis

The binding of sulfamethazine in plasma from the six patients is shown in fig 5 As expected the sulfonamide binding was less than normal (77 %

Table 7

Binding data on anephric patients with sulfamethazine (SMT)

Patient (sex, age)	% SMT bound (100 μg/ml)		Binding constant (× 10³ M)		Binding sites		Albumin	Total protein
	Pre	Post	Pre	Post	Pre	Post	%	%
No 1 L P (M, 30)	69	47	2 8	1 2	2	2	3 5	6 1
No 2 M W (F, 41)	55	50	3 7	3 1	1 7	1 7	2 6	5 7
No 3 S R (M 39)	54	56	2 4	2 4	2	2	3 2	5.5
No 4 B B (F 16)	66	68	4 2	4 2	1 7	1 6	3 3	6 0
No 5 R W (M, 16)	57	61	3 6	2 8	1 5	1 5	3 5	6 6
No 6 R M (M, 45)	53	36	2 6	1 6	1 3	1 3	3 8	6 4
Controls (3)	77		3 3		2		4 2	7 0

deficiency but the exact mechanism(s) has not been identified (ANTON 1968, BUTTNER *et al* 1964, CLAUSEN 1966, GOLDSTEIN 1949, SCHOLTAN 1961, SOLOMON *et al* 1968, WISHINSKY *et al* 1962) Since sulfonamides are mainly bound to plasma albumin (ANTON 1960, CLAUSEN 1966, DAVIS 1943, GOLDSTEIN 1949, SCHOLTAN 1961) it would seem that a decrease in this protein should result in a decrease in binding In general this is true, but it is not a proportionate relationship Several investigators reported that a decrease in albumin concentration in disease could account for a proportionate decrease in the binding of a drug (BLONDHEIM 1955, REYNOLDS & CLUFF 1960, ROLINSON & SUTHERLAND 1965, WISHINSKY *et al* 1962) but we as well as others (SCHOLTAN 1961), found that these two characteristics were not directly related We found a decrease in albumin and sulfonamide binding in all the anephric patients but it was not a proportionate relationship Additional evidence for this comes from the results with randomly selected hospitalized patients, one of whom had a plasma albumin concentration of 2 6 %, the lowest in the study, and yet his sulfonamide binding was at the lower limits of normal The lack of a direct correlation with albumin concentration may be due to binding to other proteins which normally are of minor importance but may become dominent in disease states, binding of sulfonamides to other proteins has now been demonstrated (CLAUSEN 1966) The binding of sulfonamides to other proteins in disease states may also explain the observation that the post-dialysis binding was less in some patients An alternative explanation could be an increase in fatty acids which are active displacing agents (SOLOMON *et al* 1968) and might be expected to increase during the stress of dialysis

The inadvertent displacement of one drug by another resulting in an undesirable response has been reported (BRODIE 1965, DESGREZ & DE TRAVERSE 1966) However, the application of this principle to rational therapeutics has not yet been accomplished From our studies with rats into this possibility (ANTON 1961), we found that the signs of toxicity from the displacing agent appeared when used at the concentration necessary for effective displacement KUNIN (1966) came to the same conclusion in the case of the penicillins, he carried out an extensive investigation *in vitro* of many potential displacing agents and then tested the most active ones for displacing activity in man

Our studies do not identify the primary defect for the deficient binding of sulfonamides in some hospitalized patients, but they suggest that disease, drugs and a decreased plasma albumin concentration are all involved It appears that some drugs by competitive antagonism decrease the affinity of the albumin molecule for the sulfonamide whereas pathology may decrease the affinity between protein and drug by altering the binding

binding of long-acting *sulfonamides was of no therapeutic consequence* However, they greatly diluted their sulfonamide containing human plasma samples in a protein-free medium for the antibacterial test, this maneuver essentially eliminated plasma protein binding as a factor in their experiments (see fig 1) Nevertheless, the therapeutic significance of the protein binding of drugs is uncertain at present This is not too surprising when one realizes that factors other than protein binding, such as the pKa and lipid solubility of the drug itself, will influence its pharmacology and consequently affect the interaction between the drug and bug Other less clearly defined characteristics, which will further confound this relationship and which were the subject of these studies, are the interindividual variability in plasma protein binding and how this might be influenced by disease and drugs

Rather than variability we found a remarkable consistency in the protein binding of several sulfonamides among healthy individuals This disagrees with SCHOLTAN (1961) who reported more variation among his normal subjects But even in his study there was less than a twofold difference in the bound fraction among his subjects It might be pointed out that the higher the binding of a drug, the greater the chance for finding in creased variability in the unbound fraction As binding approaches 100 % small errors in the bound fraction will be reflected by an inordinately *large increase in the unbound fraction Further support* for binding con sistency among healthy individuals came from a literature survey in which remarkably good agreement was found between investigators from dif ferent countries on the binding of sulfonamides in man and various labo ratory animals

There is also some disagreement as to the variability in the binding of penicillin in man (BOND *et al* 1963, ROLINSON & SUTHERLAND 1965) WARREN (1965/66) summarized the binding data on 12 different penicillins from several investigators and concluded that the results were widely divergent On the contrary, I interpret the data as being in relatively good agreement (with the exception of nafcillin) considering that different me-thods were used and there was less than a twofold range in the unbound fraction with any one of the penicillins Furthermore, 3 investigators not only were in fairly good agreement as to the degree of binding of 5 peni cillins but they also listed them in the same relative order

In contrast to the lack of unanimity as to binding consistency among healthy individuals, all agree that binding is decreased in disease (ANTON 1968, BUTTNER *et al* 1964, GOLDSTEIN 1949, REYNOLDS & CLUFF 1960, SCHOLTAN 1961) An increase in certain circulating metabolites, e g, bili-rubin, fatty acids, drugs, decreased albumin concentration, and altered protein structure have been suggested as possible causes for the binding

n, C M Clinical pharmacology of the new penicillins. I The importance of serum protein binding in determining antimicrobial activity and concentration in serum *Clin Pharmacol Therap* 1966, **7**, 166–179 and 180–188
sen, S T, O Ovsthus & J Boe Antibacterial activity of long acting sulfonamides. *Acta Med Scand.* 1963, **173**, 707–717
bould, B B & R Kilpatrick Long acting sulphonamides and protein binding *Lancet* 1960 **1**, 887–891
nolds, R C & L E Cluff Interaction of serum and sodium salicylate changes during acute infections and its influence on pharmacological activity *Bull J Hopkins Hosp* 1960, **107**, 278–290
erts, C E, Jr, D M Perry, H A Kuharic & W M M Kirby Demethylchlortetracycline and tetracycline A critical comparison *In vitro* activity, serum concentration, and effect of protein binding *Arch Int Med* 1961, **107**, 204–211
nson, G N & R Sutherland The binding of antibiotics to serum proteins *Brit J Pharmacol* 1965, **25**, 638–650
oltan, W Die Bindung der Langzeit sulfonamide und die Eiweisskorper des Serums *Arzneimittel Forsch* 1961, **11**, 707–720
mon, H M, J J Schrogie & D Williams The displacement of phenylbutazone 14C and warfarin 14C from human albumin by various drugs and fatty acids *Biochem Pharmacol* 1968, **17**, 143–151
rren, G H The prognostic significance of penicillin serum levels and protein binding in clinical medicine A review of current studies. *Chemotherapia* 1965/66, **10**, 339–358
binsky H, E J Glasser & S Perkal Protein interaction of sulfonylurea compounds *Diabetes* 1962, **11**, Suppl 18–25

sites qualitatively and quantitatively More detailed binding studies with protein fractions from healthy and ill patients in the presence and absence of other drugs should help to resolve this problem This could result not only in a better understanding of the basis for some of the side effects of drugs, but also could lead to the design of useful displacing agents

REFERENCES

Anton, A H The relation between the binding of sulfonamides to albumin and their antibacterial efficacy *J Pharmacol* 1960, **129**, 282–290

Anton, A H A drug induced change in the distribution and renal excretion of sulfonamides *J Pharmacol* 1961, **134**, 291–303

Anton, A H The effect of disease, drugs and dilution on the binding of sulfonamides in human plasma *Clin Pharmacol Therap* 1968, **9**, 561–567

Anton, A H & J J Boyle Alteration of the acetylation of sulfonamides by protein binding, sulfinpyrazone and suramin *Canad J Physiol Pharmacol* 1964, **42**, 809–817

Batterman, R C, L F Tauber & M E Bell Long acting sulfonamides *In vitro* correlation in man of protein binding serum concentration and antimicrobial activity *Curr Therap Res* 1966, **8**, 75–84

Blondheim, S H The relationship between the albumin concentration of serum and its dye binding capacity *J Lab Clin Med* 1955, **45**, 740–747

Bond J M, J W Lightbrown, M Barber & P M Waterworth A comparison of four phenoxypenicillins *Brit Med J* 1963, **2**, 956–961

Bratton, A C & E K Marshall, Jr A new coupling component for sulfanilamide determination *J Biol Chem* 1939, **128**, 537–550

Brodie, B B Displacement of one drug by another from carrier or receptor sites *Proc Roy Soc Med* 1965, **58**, 946–955

Brodie, B B & C A M Hogben Some physicochemical factors in drug action *J Pharm Pharmacol* 1957, **9**, 345–380

Buttner, H, F Portwich, E Manske & N Staudt Zur Pharmakokinetik von Sulfonamiden unter pathologischen Bedingungen *Klin Wschr* 1964, **42**, 103–108

Clausen, J Binding of sulfonamides to serum proteins Physicochemical and immunochemical studies *J Pharmacol* 1966 **153**, 167–175

Davis, B D The binding of sulfonamide drugs by plasma proteins A factor in determining the distribution of drugs in the body *J Clin Invest* 1943, **22**, 753–762

Desgrez, P & P M De Traverse (Eds) *Transport function of plasma proteins* West European Symposia on Clinical Chemistry Vol 5, 1966 Elsevier Publishing Co

Goldstein, A The interaction of drugs and plasma proteins *Pharmacol Rev* 1949, **1**, 102–165

Klein, J O & M Finland The new penicillins *New Eng J Med* 1963 **269**, 1074–1082

Klotz, I M, F M Walker & R B Pivan The binding of organic ions by proteins *J Am Chem Soc* 1946, **68**, 1486–1490

Kruger Thiemer, E E Wempe & M Topfer Die antibakterielle Wirkung des nicht eiweissgebundenen Anteils der Sulfanilamide im menschlichen Plasmawasser *Arzneimittel Forsch* 1965, **15**, 1309–1317

Kunin, C M Clinical pharmacology of the new penicillins. I The importance of serum protein binding in determining antimicrobial activity and concentration in serum *Clin Pharmacol Therap* 1966, 7, 166–179 and 180–188

Madsen, S T, O Ovsthus & I Boe Antibacterial activity of long acting sulfonamides *Acta Med Scand* 1963, 173, 707–717

Newbould, B B & R. Kilpatrick Long acting sulphonamides and protein binding *Lancet* 1960, 1, 887–891

Reynolds, R C. & L. E. Cluff Interaction of serum and sodium salicylate changes during acute infections and its influence on pharmacological activity *Bull J Hopkins Hosp* 1960 107, 278–290

Roberts C E, Jr, D M Perry, H A. Kuharic & W M M Kirby Demethylchlortetracycline and tetracycline A critical comparison *In vitro* activity, serum concentration, and effect of protein binding *Arch Int Med* 1961, 107, 204–211

Rolinson G N & R. Sutherland The binding of antibiotics to serum proteins *Brit J Pharmacol* 1965, 25, 638–650

Scholtan, W Die Bindung der Langzeit sulfonamide und die Eiweisskorper des Serums. *Arzneimittel Forsch* 1961, 11, 707–720

Solomon, H M, J J Schrogie & D Williams The displacement of phenylbutazone 14C and warfarin 14C from human albumin by various drugs and fatty acids *Biochem Pharmacol* 1968, 17, 143–151

Warren G H The prognostic significance of penicillin serum levels and protein binding in clinical medicine A review of current studies. *Chemotherapia* 1965/66, 10, 339–358

Wishinsky H., E J Glasser & S. Perkal Protein interaction of sulfonylurea compounds *Diabetes* 1962, 11, Suppl 18–25

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Anton, A H The relation between the binding of sulfonamides to albumin and their antibacterial efficacy *J Pharmacol* 1960, **129**, 282–290

Anton, A H A drug induced change in the distribution and renal excretion of sulfonamides *J Pharmacol* 1961, **134**, 291–303

Anton, A H The effect of disease, drugs and dilution on the binding of sulfonamides in human plasma *Clin Pharmacol Therap* 1968, **9**, 561–567

Anton, A H & J J Boyle Alteration of the acetylation of sulfonamides by protein binding, sulfinpyrazone and suramin *Canad J Physiol Pharmacol* 1964, **42**, 809–817

Batterman, R C, L F Tauber & M E Bell Long acting sulfonamides *In vitro* correlation in man of protein binding, serum concentration and antimicrobial activity *Curr Therap Res* 1966, **8**, 75–84

Blondheim, S H The relationship between the albumin concentration of serum and its dye binding capacity *J Lab Clin Med* 1955, **45**, 740–747

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Bratton, A C & E K Marshall, Jr A new coupling component for sulfanilamide determination *J Biol Chem* 1939, **128**, 537–550

Brodie, B B Displacement of one drug by another from carrier or receptor sites *Proc Roy Soc Med* 1965, **58**, 946–955

Brodie, B B & C A M Hogben Some physicochemical factors in drug action *J Pharm Pharmacol* 1957, **9**, 345–380

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Davis, B D The binding of sulfonamide drugs by plasma proteins A factor in determining the distribution of drugs in the body *J Clin Invest* 1943, **22**, 753–762

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Goldstein, A The interaction of drugs and plasma proteins *Pharmacol Rev* 1949 **1**, 102–165

Klein, J O & M Finland The new penicillins *New Eng J Med* 1963 **269** 1074–1082

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human adults The DPH-binding was measured in heparinized plasma with an *in vitro* ultrafiltration technique as described by BORGÅ *et al* (1969) and LUNDE *et al* (1970) utilizing ^{14}C-labelled DPH The degree of binding was calculated as the quotient of the radioactivity in the ultrafiltrate and in the bag (prepared from cellophane dialysis tubings)

At room temperature, pH 7 4 and with therapeutic concentrations of DPH (16 μg/ml) the bound fraction in 15 volunteers (8 males, 7 females) was 92 6 ± 0 7 per cent, indicating small interindividual differences No significant sex differences were detected Similar data were obtained in heparin plasma from the blood bank and in 4 DPH-treated adult patients with grand mal epilepsy At 37° the unbound fraction of DPH was increased by some 60 per cent, as compared to the values detected at room temperature The values for DPH binding obtained at 37° corresponds well to the actual relative concentrations of DPH (1 10) detected in cerebrospinal fluid and plasma from chronically treated patients (findings in this laboratory, unpublished)

Since patients with epilepsy often are treated with other anticonvulsants or additional drugs, the effect on DPH binding of 15, mostly acidic drugs was tested Among this series of drugs, added *in vitro* in concentrations which may be obtained during therapy, only salicylic acid, sulfafurazole and phenylbutazone caused marked decrease in DPH binding The unbound fraction of DPH increased more than two fold in the presence of salicylic acid in a concentration of 3 mM (414 μg/ml) When phenylbutazone was present in a concentration of 0 3 mM (91 μg/ml) the unbound fraction of DPH increased from 6 7 per cent Paraaminosalicylic acid, chlorothiazide and sulfamethoxypyridazine also exerted a slight, although evident displacing effect on DPH binding Of the anti-epileptic drugs tested (phenobarbital (phenemateum NFN), acetazolamide, carbamazepine, ethosuximide, sulthiam) only acetazolamide in high concentrations (45 5 μg/ml) caused a significantly reduced DPH binding All of these experiments were performed at room temperature

When plasma was diluted with 0 15 M phosphate buffer (pH 7 4) keeping the concentration of DPH constant (16 μg/ml), the bound fraction of DPH in per cent decreased successively with increasing dilution An almost identical curve was obtained when plasma and DPH were diluted to the same extent Under the latter conditions the *concentration* (μg/ml) of unbound DPH was nearly constant within a wide range of dilution

In the former situation, keeping the DPH-concentration constant, the concentrations of bound and unbound DPH changed in a reciprocal manner These data are similar to those obtained by ANTON (1968) with sulphonamides.

The experiments with dilution of both plasma and DPH may to some

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Plasma Protein Binding of Diphenylhydantoin in Man

By

Per Knut M. Lunde

Abstract The plasma protein binding of diphenylhydantoin (DPH) in heparinized plasma from adult volunteers, DPH treated adult patients with epilepsy, normal and hyperbilirubinaemic newborn infants was investigated, using an ultrafiltration technique (^{14}C labelled DPH) At room temperature (16 µg DPH/ml plasma) the bound fraction of DPH in 15 adult volunteers and 13 normal infants (cord blood) was 92 6 ± 0 7 and 89 4 ± 1 4 %, respectively At 37° the unbound fraction of DPH was increased by some 60 % Similar data were obtained in adult patients Among a series of 15, mostly acidic drugs added *in vitro* in concentrations which may be obtained during therapy, only salicylic acid, sulfafurazole and phenylbutazone caused marked decrease in DPH binding Dilution experiments showed different patterns with regard to the bound and the unbound *amounts* (µg/ml) of DPH, depending on whether plasma was diluted only (constant DPH concentration) or both DPH and plasma were diluted to the same extent In a group of 20 hyperbilirubinaemic newborn infants a correlation existed between the unbound fraction of DPH and the total concentration of plasma bilirubin A competition between DPH and bilirubin for similar binding sites on the albumin molecule is suggested

Key words Diphenylhydantoin – plasma proteins – kinetics – hyperbilirubinaemia

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According to BUCHTAL *et al* (1960) a correlation exists between the total plasma concentration of diphenylhydantoin (phenytoinum NFN) (DPH) and its therapeutic effects in patients with epilepsy If it is correct that only the unbound fraction of a drug is therapeutically active we would then expect this fraction of DPH to be fairly constant in various human individuals TRIEDMAN *et al* (1960), however, found that the unbound fraction of DPH in plasma from patients with epilepsy varied from 4 1 to 31 4 per cent On the other hand, GANSHORN & KURZ (1968) reported much less variations in the unbound fraction of DPH in plasma from human adults (14 2 ± 1 per cent)

With these contradictory results in mind we felt it necessary to study the plasma protein binding of DPH in untreated as well as DPH-treated

plasma bilirubin and plasma albumin concentration the correlation coefficient became greated This strenghtens the idea not only that albumin is an important plasma protein for binding of DPH, but also that DPH and bilirubin may compete for similar binding sites on the albumin molecule

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REFERENCES

Anton, A. H.. The effect of disease, drugs and dilution on the binding of sulphonamides in human plasma. *Clin Pharmacol Therap* 1968, 9, 561–567

Borgå, O., D L. Azarnoff, G Plym Forshell & F Sjoqvist Plasma protein binding of tricyclic antidepressants in man. *Biochem Pharmacol* 1969, 18, 2135–2143

Buchthal F, O Svensmark & P J Schiller Clinical and electroencephalographic correlations with serum levels in diphenylhydantoin. *Arch Neurol* 1960, 2, 624–630

Cederlof I. O., P K. M Lunde & F Sjoqvist Abnormal pharmacokinetics of diphenylhydantoin in a case of uremia. *Lancet* 1970, 2, 831–832.

Gansborn, A. & H Kurz. Unterschiede zwischen der Proteinbindung Neugeborener und Erwachsener und ihre Bedeutung fur der pharmakologische Wirkung. *Arch Pharmak Exper Pathol* 1968, 260, 117–118

Jalling, B., L. O Boreus, A. Rane & F Sjoqvist Plasma concentrations of diphenylhydantoin in young infants *Pharmacol Clin* 1970, 2, 200–202.

Lunde, P K. M., A. Rane, S J Yaffe, L. Lund & F Sjoqvist. Plasma protein binding of diphenylhydantoin in man Interaction with other drugs and the effect of temperature and plasma dilution. *Clin Pharmacol & Therap* 1970, 11, 846–855

Odell G B Studies in kernicterus. I. The protein binding of bilirubin. *J clin Invest* 1959, 38 823–833

Rane, A., P K. M. Lunde, B. Jalling, S J Yaffe & F Sjoqvist Plasma protein binding of diphenylhydantoin in normal and hyperbilirubinemic newborn infants. *J Pediatrics* 1971 78 877–882.

Triedman, H. M., R. A. Fishman, & M D Yahr Determination of plasma and cerebrospinal fluid levels of dilantin in the human. *Trans Amer Neurol Ass* 1960 85 166–170

extent correspond to the situation in an outbled patient with subsequent substitution of the lost fluid with plasma expanders The fact that the free fraction of DPH increases with increasing dilution may also be of clinical and toxicological significance (LUNDE *et al* 1970)

In one patient suffering from serious uremia (phenacetin abuse for 20 years) and having epileptic seizures some interesting pharmacological observations were made (CEDERLOF *et al* 1970) When being on a dosage regimen of 0 2 g DPH daily this patient still had frequent seizures Plasma analyses revealed very low concentrations of DPH (0 75–1 2 μg/ml) Further investigations indicated that this might be due to rapid metabolism (hydroxylation) of this and related drugs, possibly a result of induction of microsomal enzymes because of his daily use of barbiturates

In addition, the unbound fraction of DPH was unusually high in this patient, namely 30 per cent, versus normally 6–8 per cent, when measured at room temperature His plasma proteins were not low enough to be the only reason for the reduced DPH binding However, at the time of investigation the patient was taking 12 additional drugs Thus, the possibility that DPH was displaced from its binding sites on plasma protein molecules by other drugs and/or endogenous substances seems reasonable

By increasing the daily dose of DPH to 0 4 g, the plasma concentration amounted to about 1 5 μg/ml During the following 4 months no seizures occurred This could be explained as being due to the relatively high unbound fraction of DPH, which would favour distribution of the drug to the brain

The importance of doing a thorough pharmacokinetic evaluation in patients where the disease itself and/or additional drug therapy may cause atypical responses to standard medication should be stressed

In plasma (cord blood) from 13 normal newborn infants the bound fraction of DPH was somewhat less than in adults (89 4 ± 1 4 per cent) when measured at room temperature This may be of therapeutic importance, and could explain why the DPH concentration obtained in plasma from young infants receiving high standard doses of the drug are markedly lower than in adults, due to an increased volume of distribution (JALLING *et al* 1970)

As shown by ODELL (1959) sulphonamides cause displacement of bilirubin from its binding sites on serum proteins This indicates that the increased tendency for development of kernicterus in hyperbilirubinaemic premature infants exposed to sulphonamide therapy is due to an increased volume of distribution of bilirubin in these patients In a group of 20 hyperbilirubinaemic newborn infants we (RANE *et al* 1971) found a correlation between the unbound fraction of DPH and the total concentration of plasma bilirubin When relating the unbound fraction of DPH to the quotient between total

tion Persons of different blood groups exist in the population at the same time, they are distinct one from another, they are not necessarily diseased, and they are maintained in the population from one generation to the next by the operation of genetic mechanisms FORD (1965) has defined genetic polymorphism as "A type of variation in which individuals with sharply distinct qualities co-exist as normal members of a population" and as "the *occurrence* together in the same habitat of two or more discontinuous forms or *phases* of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation"

There are genetic polymorphisms for many properties, e g. *morphological*, erythrocytic antigens, tissue antigens, haemoglobins and various *enzymes* Since pharmacological events depend upon enzyme action it is apparent that the concept of polymorphism has a bearing upon *pharmacology*

A position on a chromosome which can be occupied by a gene is called a locus and alternative genes which can occupy this position are termed alleles Hence if a locus is termed A and the two alleles are 1 and 2, there are three types of individual A_1A_1, A_1A_2 and A_2A_2 In a quantitative character, if one locus has an overwhelming effect on the variability then two or more modes will be produced in the frequency distribution histogram Hence the effects of alleles at that locus can be recognised This was the situation with the tall and short peas of MENDEL (1865) and the same phenomenon allows recognition of polymorphism in quantitative pharmacological data

Pharmacogenetic polymorphisms of drug metabolism will now be discussed There are other pharmacogenetic polymorphisms in the actions of drugs on physiological systems, e g ocular tension and clotting mechanisms but they do not fall within the compass of this talk.

Polymorphisms of drug metabolism can be subdivided into firstly those in which all phenotypes are common and, secondly, those in which one phenotype is rare (EVANS 1969a)

Following the ingestion of 10 mg isoniazid per kg body weight the plasma concentration of the unchanged drug is bimodal Family studies have revealed that this is a genetic polymorphism Slow metabolizers of the drug who have high blood levels *are Mendelian recessives*, whereas *rapid metabolizers* with low blood levels are the dominants This means that *there are two genotypes amongst the rapid* recessives, the heterozygote and the homozygote it resembles The character is controlled by two allelic genes (EVANS *et al* 1960)

Proof regarding the enzyme controlled by these allelic genes was obtained in two ways

1 Sulfamethazine (Sulfadimidinum NFN) is polymorphically acetylated,

11*

Department of Medicine, University of Liverpool, Liverpool, England

Inter-Individual Differences in Metabolism of Drugs: The Role of Genetic Factors

By

David Alan Price Evans

Abstract Variability is a frequent finding in pharmacology, and may have a unimodal or polylmodal distribution Both types of variation are analysable by genetic techniques Experiments reveal that the variability between persons in their capacity to metabolize drugs is largely genetically determined Persons who have a greater ability to metabolize a drug may be protected from adverse reactions, but may also not receive therapeutic benefits as compared with those less well endowed, when fixed dosage regimes are utilized

Key words Drug metabolism – variability – clinical pharmacology – toxic effects – adverse effects – pharmacogenetics

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Variability is a property which is apparent in the presentations of many speakers at this Conference, and it makes the results of some pharmacological studies somewhat difficult to interpret Although variability can be regarded in a sense as a pharmacokinetic nuisance, it happens to be the raw material of genetics

Consider a hypothetical experiment in which a standard dose of a drug is given in a standard manner to a large number of healthy persons A measurement can then be made of some aspect of metabolism or of pharmacologic effect From these measurements frequency distributions can be constructed, and it can be envisaged that they could be of two types Firstly, a unimodal curve of continuous variation, and secondly a polymodal (e g bimodal or trimodal) distribution could be observed (EVANS 1962)

These distributions can both be analysed by means of genetic techniques

Polymodal distributions

Polymodal variability is frequently a clue to the existence of a genetic polymorphism This term is perhaps best understood by reference to the ABO blood groups, which are easily detected by means of two antisera Anti-A and Anti-B, giving the characteristic pattern of agglutination reac-

tion Persons of different blood groups exist in the population at the same time, they are distinct one from another, they are not necessarily diseased, and they are maintained in the population from one generation to the next by the operation of genetic mechanisms FORD (1965) has defined genetic polymorphism as "A type of variation in which individuals with sharply distinct qualities co exist as normal members of a population" and as "the occurrence together in the same habitat of two or more discontinuous forms or *phases* of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation."

There are genetic polymorphisms for many properties, e g morphological, erythrocytic antigens, tissue antigens, haemoglobins and various enzymes *Since pharmacological events depend upon enzyme action* it is apparent that the concept of polymorphism has a bearing upon pharmacology

A position on a chromosome which can be occupied by a gene is called a locus, and alternative genes which can occupy this position are termed *alleles* Hence if a locus is termed A and the two alleles are 1 and 2, there are three types of individual A_1A_1, A_1A_2 and $A A_2$ In a quantitative character, if one locus has an overwhelming effect on the variability then two or more modes will be produced in the frequency distribution histogram Hence the effects of alleles at that locus can be recognised This was the situation with the tall and short peas of MENDEL (1865) and the same phenomenon allows recognition of polymorphism in quantitative pharmacological data

Pharmacogenetic polymorphisms of drug metabolism will now be discussed *There are other pharmacogenetic polymorphisms in the actions of* drugs on physiological systems, e g ocular tension and clotting mechanisms but they do not fall within the compass of this talk.

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Proof regarding the enzyme controlled by these allelic genes was obtained in two ways

1 Sulfamethazine (Sulfadimidinum NFN) is polymorphically acetylated,

and acetylation is the only biotransformation of importance which this drug undergoes (EVANS & WHITE 1964)

2 Patients were phenotyped prior to abdominal operations and at operation a small liver biopsy was obtained The biopsies were shown to have very different activities of N acetyl transferase causing the acetylation of isoniazid and sulfamethazine from acetyl CoA Liver tissue from rapid acetylators was shown to be very active, and tissues from slow acetylators exhibited only low activity of the enzyme (EVANS & WHITE 1964)

Hence the enzyme controlled by these allelic genes was shown to be liver N-acetyl transferase

The consequences of this polymorphism can be gauged from the following In one series of healthy subjects given a single dose of sulfamethazine, the rapid acetylators excreted 27 % of the dose in the urine in the 8 hours following drug ingestion, whereas the slow acetylators only excreted 15 % of the dose in the same period Obviously on repeated dosing the cumulative effect of the genetic difference would be quite large (EVANS 1969b)

Table 1

Results of intermittent dosage regimens in tuberculosis patients of both acetylator phenotypes

Regimen	Frequency of drug administration	Acetylator phenotype	Numbers of patients Favourable response	Poor response
Streptomycin 1 g Isoniazid 14 mg per kg	twice weekly	slow	75	7
		rapid	33	3
ditto	once weekly	slow	49	11
		rapid	33	22
ditto	daily for first month then once weekly	slow	61	3
		rapid	28	9
ditto plus Pyrazinamide 90 mg per kg	once weekly	slow	58	9
		rapid	18	16

Data from the Madras Tuberculosis Chemotherapy Centre by the kind permission of Dr D A. Mitchison.

Favourable response = sputum conversion i e disappearance of tubercle bacilli see *Brit med J* (1967) iv, 230–1 and *Lancet Annotation* (1967) ii 977 8

Four items of information reveal the therapeutic significance of this polymorphism

1 The response of tuberculosis to treatment. I am indebted to Professor D A. Mitchison of the Royal Post graduate Medical School, London, for information on these experiments which were performed at the Madras Tuberculosis Chemotherapy Centre All subjects in four separate experiments had isoniazid sensitive tubercle bacilli and had not been previously treated for their tuberculosis The results were assessed by the disappearance of tubercle bacilli from the sputum after 9 to 12 months of therapy The results are shown in table 1 The rapid acetylators fare significantly worse than the slow acetylators when the dosage frequency is reduced from twice weekly to once weekly
2 Polyneuritis has been shown to occur significantly more frequently in slow acetylators than in rapid acetylators when a malnourished population is treated with conventional dosages of isoniazid. (DEVADATTA *et al* 1960)
3 Hydrallazine has a hydrazine side chain resembling isoniazid Evidence has been found from the study of human liver *in vitro* that this drug is also polymorphically acetylated (EVANS & WHITE 1964) PERRY *et al* (1967) have shown that slow acetylators are more prone than rapid acetylators to develop antinuclear antibodies (ANA) on hydrallazine therapy Further all 12 patients with the hydrallazine systemic lupus erythematosus like syndrome which these authors studied were white slow acetylators with ANA.
4 Phenelzine also has a hydrazine side chain EVANS *et al* (1965) have shown that slow acetylators are more prone than rapid acetylators to show severe side effects on therapy with phenelzine (EVANS *et al* 1965)

An example will now be considered of a pharmacogenetic polymorphism of drug metabolism in which one phenotype is rare This was described by SHAHIDI (1967 & 1968) in a female patient who developed serious methaemoglobinaemia whilst ingesting phenacetin This patient was shown to have a relative inability to de-ethylate phenacetin, and in consequence metabolized more of the drug to the toxic compounds phenetidine, hydroxyphenacetin, and hydroxy phenetidine These compounds were responsible for the methaemoglobinaemia. The patient's sister was shown to have the same phenotype, whereas the remaining relatives who were tested were shown to possess the usual capacity to de-ethylate phenacetin

Unimodal distributions

The finding of a continuous unimodal distribution means that genetic analysis along the lines described above is not appropriate

and acetylation is the only biotransformation of importance which this drug undergoes (EVANS & WHITE 1964)

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to be repeatable, and to correlate with the steady-state plasma concentration of PBZ determined on a separate occasion. These half lives were also correlated with height, shorter subjects having shorter halflives, and therefore height adjusted values could be computed.

The height adjusted post-phenobarbitone $\log_{10}$ plasma PBZ half lives had a "heritability" of 0.65 ± S.E.M. 0.21 (fig. 1). Therefore for this drug by means of this particular test 65 % of the variability in metabolism is due to genetic factors.

Factors such as disease, differing routes of administration, etc., could increase V_E but V_G is a component of variability which is built into the population.

BRUCK *et al.* (1954) showed that various toxic effects were more common in patients whose blood PBZ concentrations exceeded 100 µg per ml than in those with lower concentrations. Subjective improvement in rheumatoid arthritic patients was more frequent when blood levels were above 50 µg PBZ per ml than when concentrations were below this level.

Conclusions

As a result of the foregoing it can be stated that people vary greatly in their ability to metabolize drugs, and that genetic factors account for

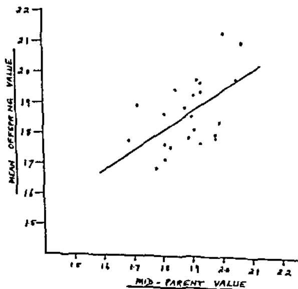


FIG. 1. Log_{10} post phenobarbitone plasma phenylbutazone half lifed adjusted to a standard height of 66 inches. Data in 24 families. From WHITTAKER & EVANS (1970)

It can be considered that a character which is highly repeatable and which has an unimodal continuous distribution is under polygenic control and that contributions of the same order of magnitude are made to the total variability by the effects of allelic genes at a number of loci Therefore the effects of allelic genes at a particular locus cannot be identified within the total variability Environmental influences can also contribute to the variability

Hence in the nomenclature of FALCONER (1960)

$$V_P = V_G + V_E \quad (1)$$

where

V_P = observed phenotypic variance
V_G = genetic contribution to variance
V_E = environmental contribution to variance

Further

$$V_G = V_A + V_D + V_I \quad (2)$$

where

V_A = is the component of variance due to the additive effects of genes
V_D = component of variance due to dominance (interaction between al leles at a locus)
V_I = component of variance due to interactions between loci

In organisms such as DROSOPHILA V_D and V_I have been shown for many characters to be very small compared to V_A

It was demonstrated by FISHER (1918) that the regression of mean offspring values for a character upon the mid parent values gave a regression coefficient = V_A/V_P, termed the heritability"

Therefore by the measurement of the character in whole families an estimate can be made of how much of the variability of the character in the population can be ascribed to genetic factors

WHITTAKER & EVANS (1970) have made this observation for phenyl butazone metabolism in man Phenylbutazone (PBZ) is known to be metabolized by microsomal hydroxylation (BURNS et al 1955) Microsomal enzymic processes are influenced by many chemical environmental agents (REMMER & MERKER 1965, BURNS & CONNEY 1965) Hence to re duce the environmental component of variance in PBZ metabolism all in dividuals were pre treated with phenobarbitone (phenemalum NFN) before their PBZ test The post phenobarbitone PBZ plasma half life was found

Perry, H M, Jr, A Sakamoto & E. M Tan Relationship of acetylating enzyme to hydralazine toxicity *Proc. Central Soc for Clinical Research* Fortieth Annual Meeting Chicago, Ill. 1967, **40**, 81–82.

Remmer, H & H J Merker Effect of drugs on the formation of smooth endoplasmic reticulum and drug metabolising enzymes *Ann N Y Acad Sci* 1965, **123**, 79–96

Shahidi N T Acetophenetidin sensitivity *Amer J Dis Child* 1967, **113**, 81–82

Shahidi, N T Acetophenetidin induced methemoglobinemia. *Ann N Y Acad Sci* 1968 **151**, 822–832.

Whittaker J A. & D A P Evans Genetic control of phenylbutazone metabolism in man *Brit med J* 1970 4 323–328

the greater part of this variability. Persons who are less able to metabolize drugs than their fellows have a greater liability to develop toxic effects On the other hand persons with more speedy metabolism of drugs than their fellows may under certain circumstances not obtain the desired therapeutic effect to the same degree

It seems probable that variability will have to receive more attention in therapeutics in the future than it has done heretofore Dosage schedules may require to be much more personalized for the individual Monitoring of plasma concentrations of drugs would seem to be a practical way of achieving the correct dose in many instances

Awareness of the biological significance of variability may result in the discovery of new human genes, knowledge of which could then be used in the study of disease

REFERENCES

Bruck, E, M E Fearnley, I Meanock & H Patley Phenylbutazone therapy Relation between the toxic and therapeutic effects and the blood level *Lancet* 1954, **1**, 225–228

Burns J J & A H Conney Enzyme stimulation and inhibition in the metabolism of drugs *Proc Roy soc Med* 1965, **58**, 955–960

Burns, J J, R K Rose, S Goodwin, J Reichenthal, E C Horning & B B Brodie The metabolic fate of phenylbutazone *J Pharmacol Exp Therap* 1955, **113** 481–489

Devadatta S, P R J Gangadharam, R H Andrews, W Fox, C V Ramakrishnan, J B Selkon & S Velu Peripheral neuritis due to isoniazid *Bull Wld Hlth Org* 1960, **23**, 587–598

Evans, D A P Pharmacogenetique *Med et Hyg (Geneve)* 1962, **20**, 905–909

Evans, D A P Genetic factors in drug therapy Chapter X, pages 166–182 *In The scientific basis of medicine Annual reviews* The Athlone Press, University of London, London, 1969a

Evans, D A P An improved and simplified method of detecting the acetylator phenotype *J med Genetics* 1969b, **6**, 405–407

Evans, D A P, K Davison & R. T C. Pratt The influence of acetylator phenotype on the effects of treating depression with phenelzine *Clin Pharmacol Therap* 1965, **6**, 430–435

Evans D A P, K A Manley & V A McKusick Human isoniazid metabolism *Brit med J* 1960, **2**, 485–491

Evans, D A P & T A White Human acetylation polymorphism *J Lab clin Med* 1964, **63**, 394–403

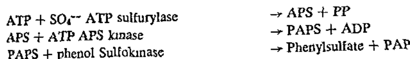
Falconer, D S *Quantitative genetics* 1960 Oliver and Boyd, Edinburgh

Fisher, R. A The correlation between relatives on the supposition of Mendelian inheritance *Trans Roy Soc (Edinburgh)* 1918, **52**, 399–433

Ford, E B *Genetic polymorphism* Faber and Farber, London, 1965

Mendel, G Versuche uber Pflanzen Hybriden Verhandlungen des naturforschenden *Vereines in Brunn* 1865, Abhandlungen, **4**, 3–47

The synthesis of the sulfate derivatives occurs as follows (WILLIAMS 1959, BOSTROM & VESTERMARK 1960)



ATP + SO_4^{--} ATP sulfurylase → APS + PP
APS + ATP APS kinase → PAPS + ADP
PAPS + phenol Sulfokinase → Phenylsulfate + PAP

Besides being interested in the short time ratio of glucuronide/sulfate ester we were mostly interested in changes of such a ratio during long term administration

Material and Methods

Adult female rats (FW 49-SPF) weighing about 200 g were used The substances were administered daily directly through the mouth into the stomach (MR 623 400 mg/kg *MR 560 450 mg/kg) Before the first* administration (urine 0) directly after (urine 1) and at the given time intervals (see figures) the animals were put into *metabolite-cages for 24 hours to collect the urine*

For the following experiments 6 beagles (3♀ 3♂) weighing 10 kg each were used. *The daily given dose (perorally, gelatine capsule) was 5 mg/kg for* MR 623 and 20 mg/kg for MR 560 To collect the 24 hr urine after specific time intervals, each animal was put into a metabolite cage

The amount of free MR substance was determined by the methylorange method (BRODIE *et al* 1947) For the determination of the total conjugates plus free MR substance the solutions were hydrolyzed in 0 5 N HCl for 1 hour at 100° before using the methylorange method The sum of glucuronide and free substance was found by a preliminary incubation with 1000 Fishman units β glucuronidase (purest bovine liver) for 5 hours at 37° Both hydrolyses were always controlled by thin layer chromatography As standards MR substances and sulfate esters were run in parallel The activity of the β glucuronidase was tested with phenolphthalein glucuronide

Results

In fig 1 the urinary excretion of MR 623 and its conjugates in 10 rats is shown The concentration in nM/ml urine is drawn against the time intervals The meaning of the different columns is explained in the figure During a daily peroral administration of 400 mg MR 623/kg the total urinary excretion is about 1 3 µmol/ml = 0 4 mg/ml The total excretion is the sum of the following columns for sulfate ester, glucuronide and free substance The average of the total substance in the urine for 24 hours is found in fig 2. The results for the 24 h urinary excretion show about 15 µmol/animal = 4 7 mg/animal This amount is fairly constant over a period of 5 weeks In contrast to the sum the sulfate ester part decreases from 92 % at the first day

From the Department of Biochemistry, C H Boehringer Sohn
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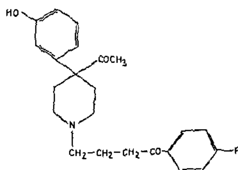
Changes in the Excretion Pattern of Drugs During Long Term Administration

By

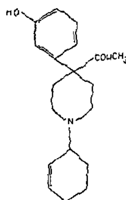
Wolfgang Pollmann and Wolf-Dieter Bechtel

A common form of metabolism of drugs is by their conjugation It was shown (GILLETTE 1963) that conjugation means mainly the reaction of phenols, carboxylic acids, long chain alcohols and primary amines with glucuronic acid or sulfate Because many drugs or their metabolites have an aromatic hydroxyl group, we used the following two analgesic substances to determine the formation of glucuronides and sulfate esters quantitatively

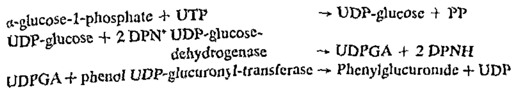
Mr 560



Mr 623



The formation of glucuronides follows the pathway (STROMINGER *et al* 1957, STOREY & DUTTON 1955)



α-glucose-1-phosphate + UTP → UDP-glucose + PP

UDP-glucose + 2 DPN⁺ UDP-glucose-dehydrogenase → UDPGA + 2 DPNH

UDPGA + phenol UDP-glucuronyl-transferase → Phenylglucuronide + UDP

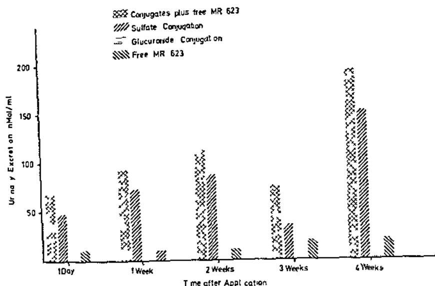


Fig 3 Urinary excretion of MR 623 and the conjugation products of 6 dogs during daily peroral administration of 5 mg/kg

Conjugates plus free MR 623
Sulfate Con ugat on
Glucuron de Conjugat on
Free MR 623
Ur nary exc etion µMol
30
20
10
1 Day
week
2 Weeks
3 Weeks
4 Weeks
T me after Appl c t on

Fig. 4. Urinary excretion of MR 623 and the conjugation products of 6 dogs during daily oral administration of 5 mg/kg

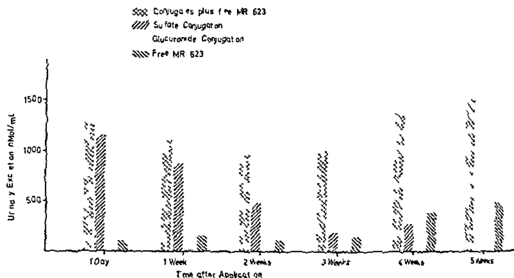


Fig 1 Urinary excretion of *MR 623 and the* conjugation products of 10 rats (♀) *during daily oral* administration of 400 mg/kg

to 0 % *after 5 weeks At the same* time the glucuronide increases from 2 % (1st day) *to 70 % after 5* weeks Moreover the free substance changes from 8 % (1st day) to 30 % *(5 weeks)*

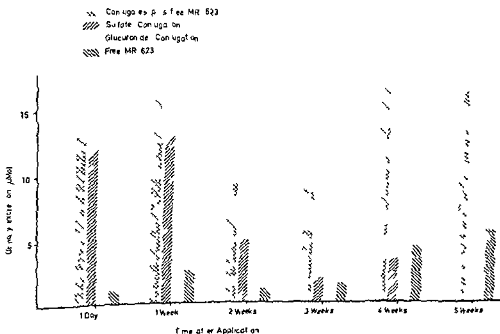


Fig. 2 Urinary excretion of MR 623 and the conjugation products of 10 *rats during* daily oral administration of 400 mg/kg

Other data were obtained with experiments on dogs Fig. 3 shows the urinary excretion of 6 beagles for MR 623 and the conjugates in nmol/ml urine The total amount of the urinary excretion in µmol/animal and day is shown in fig 4 In this case not only the sum of excretion products but also the sulfate ester and glucuronide parts are constant In contrast to the rat here is no change in the conjugate ratio

Analogous data were obtained with MR 560 Fig 5 shows the concentration fig 6 the total amount of the urinary excretion of MR 560 in 10 rats The experimental time range was now 13 weeks Again the sum of the excretion is almost constant but the sulfate ester part decreases from about 100 % (1st day) to 25 % (4 weeks) and finally to 3 % after 13 weeks In parallel the glucuronide increases from 0 % (1st day) to 75 % (13 weeks)

The experiments with dogs and MR 560 are shown in fig 7 (nmol/ml urine) and fig 8 (µmol/animal and day) As the figures show MR 560 is almost all excreted as the sulfate ester

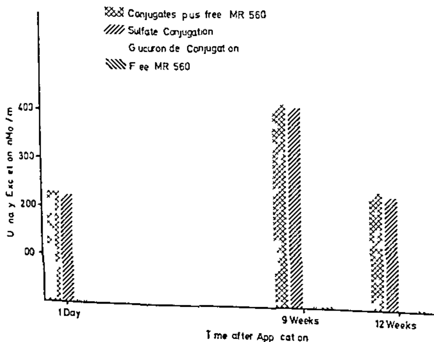


Fig. 7 Urinary excretion of MR 560 and the conjugation products of 6 dogs during daily peroral administration of 20 mg/kg.

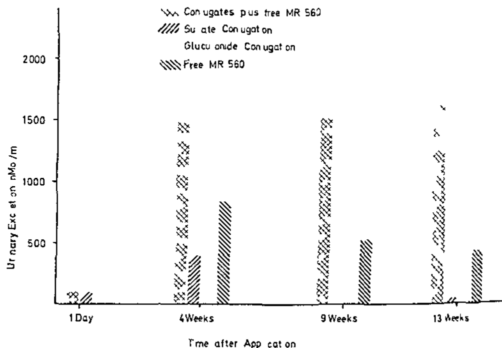


Fig 5 Urinary excretion of MR 560 and the conjugation products of 10 rats during daily oral administration of 450 mg/kg

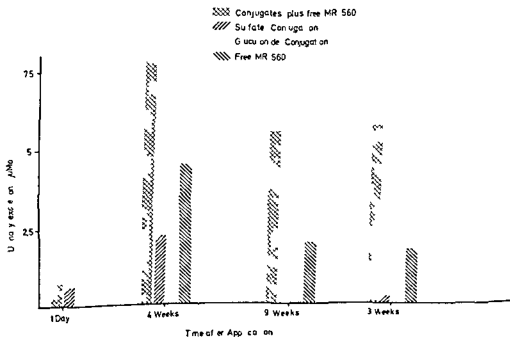


Fig 6 Urinary excretion of MR 560 and the conjugation products of 10 rats during daily oral administration of 450 mg/kg

a conjugation change It is not very likely that the "active sulfate" pool has become insufficient New experiments with lower doses gave the same change in rats The fact that parallel to the glucuronide also the amount of free substance increases (fig. 1) shows that induction of the glucuronide forming system is not the sole explanation It would be expected that the free substance is excreted as the sulfate ester Therefore the most conceivable explanation at the moment would be that an induction of the glucuronide system and a simultaneous depression of the sulfate system occurs

Summary

The urinary excretion patterns of the two analgesic substances MR 623 and MR 560 were studied in rats and dogs The substances are mainly excreted as their conjugates, i e sulfate esters or glucuronides In contrast to the constant results obtained with experiments on dogs, an extreme decrease in the sulfate ester with a correspondingly high increase in glucuronide during the long term administration (5 - 13 weeks) was found in rats That means a species specific change in metabolism has occurred

REFERENCES

Bostrom H. & A Vestermark Studies on ester sulphates *Acta physiol Scand* 1960, *48* 88–94

Brodie B B S Udenfriend W Dill & Th. Chenkin The estimation of basic organic compounds in biological material *J Biol Chem* 1947, *168* 319–325

Gillette J R. Metabolism of drugs and other foreign compounds by enzymatic mechanisms *Progress in Drug Research* 1963, *6*, 11–73

Storey I D E. & G J Dutton Uridine compounds in glucuronic acid metabolism. *Biochem J* 1955 *59* 279–288

Williams, R. T *Detoxication mechanisms* 2nd ed John Wiley and Sons Inc., New York, 1959 p 279

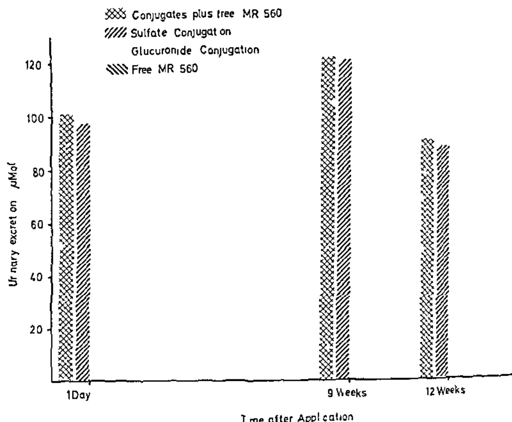


Fig 8 Urinary excretion of MR 560 and the conjugation products of 6 dogs during daily oral administration of 20 mg/kg

Discussion

From the experiments, MR 623 and MR 560 after resorption by the rat or dog are mainly excreted as their conjugates This behaviour would be expected from the phenolic hydroxylgroup in both substances The surprising fact was, however, that in the rat both drugs are first excreted as their sulfate ester, but after daily administration for some weeks the substances were mainly excreted as glucuronides Comparison of figs 1 and 3 shows that in contrast to MR 560, MR 623 reaches its steady state after one day The experiments with dogs show that no similar change of metabolism occurs during long term administration This means that there is a species specific change in the metabolism of the two drugs Such changes are not only important for fundamental research but might also be important for practical reasons If the sulfate ester for instance has a low solubility, a decrease of the ester with a parallel increase of the glucuronide would be a safety factor for long term administration

At the present time it is almost impossible to give a full explanation for such

Table 1

Amphetamine clearance from plasma after an oral dose of 15 mg (BECKETT *et al* 1969)

Condition of urine	Clearance of amphetamine from plasma (ml/min.)	
	Subject 1	Subject 2
Acid pH (< 5 2)	432–539	242–387
Fluctuating pH	16–115	41–64
Glomerular filtration (creatinine clearance)	125	126

whether inter individual differences are due to disease states or pharmacogenetic factors

The importance of urinary pH control in studies involving acidic or basic drugs is now well established (BECKETT 1966, BECKETT *et al* 1969, VREE *et al* 1970) A recent study conducted among West Africans by WESLEY

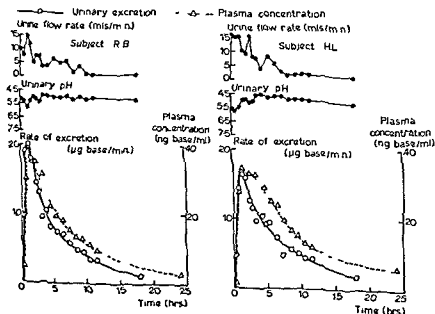


Fig. 1 Plasma levels (--△--) and urinary excretion rates (—○—) in two subjects after an oral dose of amphetamine (15 mg)

From the Department of Pharmacy,
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Pharmacokinetic Studies on Pentazocine

By

Mervyn Mitchard

It is 30 years since Professor Torsten Teorell published his compartment concept of drug distribution in a living organism (TEORELL 1937) and almost 20 years since Professor Dost introduced the term pharmacokinetics to describe the study and analysis of drug distribution data (DOST 1953). Subsequently, this term has become widely used and is becoming increasingly misused.

Pharmacokinetic data is obtained from studies in which drug and/or metabolite concentration time curves are obtained for specific 'compartments" of the organism, the most important of which are the blood and urine.

The slow development of the subject, in which significant advances have been made only during the latter part of the last decade, has been due to a lack of sensitive, specific analytical methods, and to an ignorance about the importance of such factors as protein binding, ion pair formation and urinary pH. Recently developed sensitive analytical procedures based on gas liquid chromatography and spectrophotofluorimetry have now made it possible to quantitatively determine drugs in the biophase at concentrations of 10^{-9} to 10^{-12} g/ml and the importance of steady state plasma levels instead of blood drug levels has now been recognised. Under the added impetus of legislation and proposed legislative changes as proposed in the Goldenthal Letter of 1968 rapid developments have been made in pharmacokinetic studies during the last two or three years.

Pharmacokinetic studies

Pharmacokinetic studies provide data from which rate constants which describe the behaviour of the drug in the living animal can be calculated. Properly conducted studies under controlled conditions yield constants which in many ways behave as fundamental characteristics of a drug. It is therefore possible to examine the influence of such factors as diet or drug formulation on drug absorption, distribution, metabolism or excretion and to determine

Table 1

Amphetamine clearance from plasma after an oral dose of 15 mg (BECKETT *et al* 1969)

Condition of urine	Clearance of amphetamine from plasma (ml/min)	
	Subject 1	Subject 2
Acid pH (< 5.2)	432 539	242 387
Fluctuating pH	16–115	41–64
Glomerular filtration (creatinine clearance)	125	126

whether inter individual differences are due to disease states or pharmacogenetic factors

The importance of urinary pH control in studies involving acidic or basic drugs is now well established (BECKETT 1966 BECKETT *et al* 1969 VREE *et al* 1970) A recent study conducted among West Africans by WESLEY

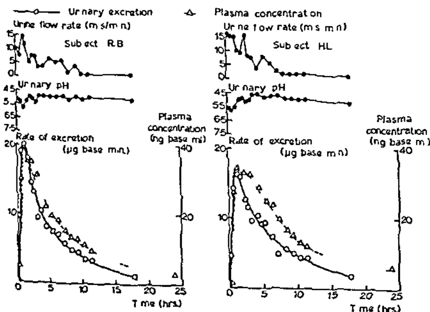


Fig. 1 Plasma levels (△) and urinary excretion rates (—○—) in two subjects after an oral dose of amphetamine (15 mg)

1

HADZIJA (1969) has established a clear relationship between diet and urinary pH in which a protein deficient carbohydrate diet resulted in a urinary pH which was never less than 7, whilst a normal protein containing diet produced a urinary pH which never exceeded 6.5. More recent studies (WESLEY-HADZIJA 1971) have shown that less than 5 % of an oral dose of amphetamine was excreted during a 16 hour period by the individuals receiving protein free, carbohydrate diets, whereas 45 to 55 % was excreted during a similar period by individuals receiving a balanced diet. The relevance of this study to the theme of this symposium, viz. "the clinical importance of inter-individual differences which determine the biological availability of drugs given to patients", will be readily apparent.

Amphetamine proved to be an ideal drug for pharmacokinetic studies. It behaves in a predictable manner under conditions in which an acidic urinary pH (< 5.2) is maintained. A high percentage of unchanged drug (< 60 %) is eliminated in urine, a large proportion of which is excreted by tubular secretion (table 1), and urinary excretion rates are directly related to plasma amphetamine levels (fig. 1), both decrease exponentially with time. Tubular reabsorption occurs under uncontrolled conditions and plasma amphetamine levels remain elevated for long periods of time (fig. 2).

Pentazocine

Pentazocine does not behave in a predictable manner nor is data, obtained from studies performed under carefully controlled conditions, always suitable for pharmacokinetic analysis.

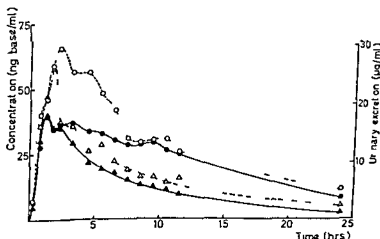


Fig. 2. Plasma levels (– – –) and urinary excretion rates (——) of amphetamine under acid controlled (▲△) and uncontrolled (●○) urinary pH.

Pentazocine is a potent analgesic having an activity reported variously as one half (KEATS & TELFORD 1964), one third (BEAVER *et al* 1968, STOELTING 1965), one fourth (BELLVILLE & FORREST 1968) and one sixth (BEAVER *et al* 1968) that of morphine The drug also has some nalorphinelike activity (JASINKI *et al* 1970) BERKOWITZ *et al* (1969) established a direct correlation between plasma pentazocine level and pain relief, the decrease in analgesic effect parallelled the fall in plasma level for a period of several hours Plasma levels of pentazocine would therefore appear to give a valid measure of drug activity

Blood and plasma levels

Peak plasma pentazocine levels of 100 to 240 mg/ml were obtained at between 15 and 60 minutes after intramuscular administration (45 ng/kg) and of 100 to 300 ng/ml at between 60 and 180 minutes after oral administration (75 mg) by BERKOWITZ *et al* (1969) and BECKETT *et al* (1970a & b) obtained blood levels of 50 to 200 ng/ml after oral administration (88 7 mg pentazocine base) and 50 to 100 ng/ml after rectal administration (100 mg) Considerable inter-subject variation was apparent (fig 3) and blood levels were shown to be route of administration dependent

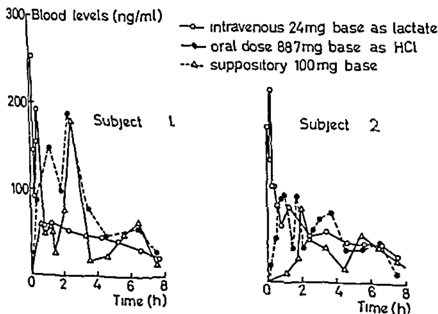


Fig. 3 Blood levels of pentazocine after various routes of administration.

Pentazocine rapidly leaves plasma and concentrates in other tissues Only 2 % of the administered drug is present in the plasma 4 minutes after an intravenous dose and only 1 % at peak plasma levels when the drug is given by other routes BECKETT *et al* (1969) calculated a similar degree of extra vascular concentration for amphetamine, only 2 5 % of the administered dose being detected in plasma 1 hour after an oral dose

Approximately 25 % of the plasma drug levels which were determined spectrophotometrically by BERKOWITZ *et al* (1969) existed as pentazocine metabolites

Excretion

Very little drug is excreted in faeces, less than 1 0 % being recovered after faecal analysis (BECKETT *et al* 1970a & b) but a significant proportion (10 to 28 %) is excreted in urine as glucuronide (BERKOWITZ & WAY 1969)

There appears to be a linear relationship between urinary excretion rates and blood pentazocine concentrations of less than 100 ng/ml (fig 4) How

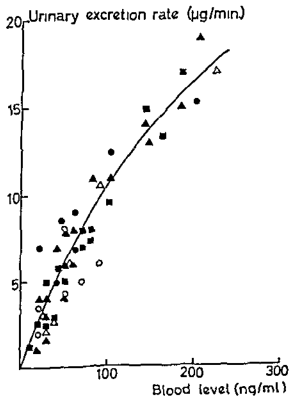


Fig 4 The relationship between urinary excretion rate of pentazocine and blood pentazocine levels under acidic conditions.

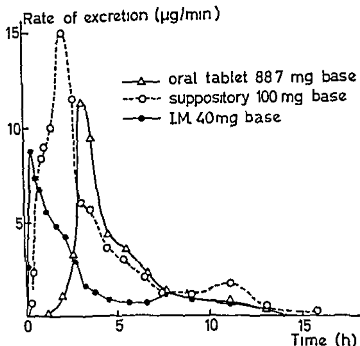


Fig. 5 Urinary excretion rates of pentazocine after intramuscular, oral and rectal administration under acidic conditions Type I

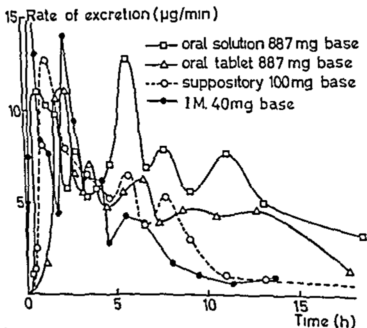


Fig. 6. Urinary excretion rates of pentazocine after intramuscular, oral and rectal administration under acidic conditions. Type II

ever, although some individuals show a typical exponential decrease in urinary excretion rate (fig 5), many individuals have atypical blood level and excretion rate profiles (fig 6) which show secondary and tertiary peaks even when a constant acidic urinary pH is maintained I have designated these "Type II" profiles Type II profiles appear to be individual dependent and not route of administration dependent These observations have not yet been explained, although they may be due to genetic differences in rate or route of metabolism

Metabolism

Pentazocine is excreted in urine as free pentazocine (5 - 10 %), glucuronide (10 - 30 %) and as polar metabolites (PITTMAN *et al* 1969, BERKOWITZ & WAY 1969), the latter have been characterised as the *cis* alcoholic and *trans* carboxylic acid metabolism produced by oxidation of the dimethyl allyl side

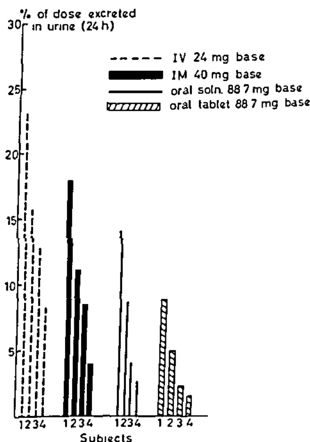


Fig 7 Urinary excretion of pentazocine in four subjects after various routes of administration under acidic conditions



Fig 8 Frequency distribution histogram of per cent unmetabolised pentazocine excreted in the urine of 15 individuals Data taken from BERKOWITZ & WAY (1969) and BECKETT *et al* (1970b)

chain (PITTMAN 1970) The decrease in plasma drug levels is therefore due primarily to metabolism The amount of drug metabolised is both route of administration and individual dependent (fig 7) BERKOWITZ *et al* (1969) suggested "that pentazocine may be more extensively metabolised when administered orally than by the parenteral route" and BECKETT *et al* (1969) have shown that when the route is changed from intravenous to intramuscular to oral solution to oral tablet, then an increase in dose of 1 5, 2 - 3 and 3 - 5 times respectively is required to produce similar blood levels These workers have shown that individuals who excrete a high percentage (10 - 14 %) of unmetabolised pentazocine also have Type II plasma and excretion rate profiles BERKOWITZ & WAY (1969) demonstrated that individuals who excrete a high percentage of unmetabolised pentazocine also excrete a high percentage *of glucuronide (21 - 28 %) as against other persons (13 - 15 %)*

The frequency distribution of the percentage of free pentazocine excreted in urine is shown in fig 8 It represents data from an extremely small population sample (15 people) and therefore is not yet significant However, it does suggest a bimodal distribution of excretors – high and low excretors which may reflect a pharmacogenetic difference in pentazocine metabolism

Summary

Three factors of interest have emerged from pharmacokinetic studies on pentazocine

1 Blood levels are route of administration dependent and therefore different doses are required to produce similar levels when given by different routes,

2. blood drug levels and urinary *excretion rate profiles* of some individuals show secondary and tertiary peaks which are related to

3 pronounced individual variation in percentages of unmetabolised pentazocine and glucuronide excreted in urine This last factor may indicate pharmacogenetically determined variations in routes and/or rates of metabolism

REFERENCES

Beaver, W T, S L Wallenstein, R W Houde & A Rogers A clinical comparison of the effects of oral and intramuscular administration of analgesics pentazocine and phenazocine *Clin Pharmacol Therap* 1968, **9**, 582–597

Beckett, A H Drug distribution and metabolism *Dansk Tidsskr Farm* 1966, **40** 197–223

Beckett, A H, J A Salmon & M Mitchard The relation between blood levels and urinary excretion of amphetamine under controlled acidic and under fluctuating urinary pH values using (^{14}C) amphetamine *J Pharm Pharmacol* 1969, **21** 251–258

Beckett, A H, J F Taylor & P Kourounakis The absorption, distribution and excretion of pentazocine in man after oral and intravenous administration. *J Pharm Pharmacol* 1970a, **22**, 123–128

Beckett, A H, P Kourounakis, D P Vaughan & M Mitchard The absorption distribution and excretion of pentazocine in man after oral, intramuscular and rectal administration *J Pharm Pharmacol* 1970b, in press

Bellville, J W & W H Forrest Respiratory and subjective effects of d and l pentazocine *Clin Pharmacol Therap* 1968, **9**, 142–151

Berkowitz, B A, J H Asling, S M Shnider & E L Way Relationship of pentazocine plasma levels to pharmacological activity in man *Clin Pharmacol Therap* 1969 **10**, 320–328

Berkowitz, B A & E L Way Metabolism and excretion of pentazocine in man *Clin Pharmacol Therap* 1969, **10**, 681–689

Dost, F H *Der Blutspiegel Kinetic der Konzentrationsablaufe in der Kreislaufflussigkeit* Leipzig 1953

Jasinki, D R., W R Martin & R D Hoeldtke Effects of short and long term administration of pentazocine in man *Clin Pharmacol Therap* 1970, **11**, 385–403

Keats, A S & J Telford Studies of analgesic drugs VIII A narcotic antagonist analgesic without psychomimetic effects *J Pharmacol Exp Therap* 1964, **143**, 157–164

Pittman K P Resi, R Cherniak, A J Merola & W D Conway Metabolism *in vitro* and *in vivo* of pentazocine *Biochem Pharmacol* 1969, **18**, 1673–1678

Pittman, K Human metabolism of orally administered pentazocine *Biochem Pharmacol* 1970, **19**, 1833–1835

Stoelting V K Analgesic action of pentazocine compared with morphine in post operative pain *Anesth & Analg* 1965, **44** 769–772

Teorell, T Kinetics of distribution of substances administered to the body *Arch int Pharmacodyn* 1937, **57**, 205–240

Vree, T B, A Th J M Muskens & J M van Rossum Some physico chemical properties of amphetamine and related drugs *J Pharm Pharmacol* 1970 in press.

Wesley Hadzija, B The effect of diet in West Africa on the pH of human urine. *J Pharm Pharmacol* 1969, **21**, 196–197

Wesley Hadzija, B A note on the influence of diet in West Africa on urinary pH and excretion of amphetamine in man *J Pharm Pharmacol* 1971 **23** 366–368

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Individual Differences in the Plasma Half-lives of Lipid Soluble Drugs in Man

By

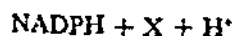
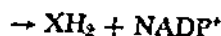
Donald S Davies and Snorre S Thorgeirsson

The duration of action of many drugs is often limited by the rate at which they are metabolized to inactive, water-soluble metabolites In the absence of drug metabolizing enzymes drugs would have to be excreted unchanged in bile or urine It has been calculated that for some protein bound, lipid soluble drugs the rate of elimination by the kidneys would be exceedingly small (BUTLER 1958) Indeed given these circumstances drugs such as chlorpromazine or thiopental (thiomebumalum NFN) would act for virtually a life time and make much of drug therapy as we now know it impossible

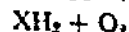
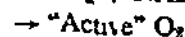
During the past fifteen years numerous studies have shown that most drugs are metabolized by a small number of reactions (GILLETTE 1963), the most common especially for lipid soluble drugs, being oxidation Oxidation of drugs occurs mainly in the liver and has been the subject of countless studies both *in vivo* and with tissue preparations *in vitro*

In the early 1950 s it was established that the microsomal fraction of the liver cell contains an enzyme system that can oxidize drugs into more polar compounds which are readily excreted (AXELROD 1955, BRODIE *et al* 1955) At present many reactions are known to be catalyzed by the liver microsomal enzymes They include N-, O- and S dealkylations as well as aliphatic and aromatic hydroxylations (GILLETTE 1966)

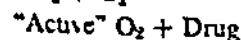
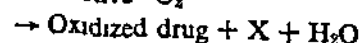
The microsomal enzymes have a requirement for reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen and have been classified as mixed function oxygenases (MASON 1957) This mechanism requires that NADPH reduce some component in microsomes The reduced component reacts with molecular oxygen to form "active" oxygen which is transferred to the drug

$$NADPH + X + H^+ \rightarrow XH_2 + NADP^+$$

$$XH_2 + O_2 \rightarrow \text{"Active" } O_2$$

$$\text{"Active" } O_2 + \text{Drug} \rightarrow \text{Oxidized drug} + X + H_2O$$

Studies in recent years have established that the component in microsomes reduced by NADPH and probably involved in the "activation' of molecular oxygen for the oxidation of drugs is cytochrome P–450 (GILLETTE 1966) Drugs which are substrates for the microsomal enzymes combine with oxidized cytochrome P–450 to give two types of changes in its visible absorption spectrum Drugs such as hexobarbital (enhexymalum NFN) and aminopyrine give "type I" difference spectra which are characterized by the appearance of a trough at 420 nm "Type II" spectral changes are produced by compounds such as aniline and are characterized by the appearance of a peak at about 430 nm (REMMER *et al* 1966)

Cytochrome P–450 is probably not reduced directly by NADPH, rather the reduction is thought to be mediated through cytochrome c reductase and possibly an unidentified carrier Thus at the present time the oxidation of a drug is thought to involve the following steps Reduction of cytochrome P 450–*drug complex by NADPH–P–450 reductase The reduced P–450 then* combines molecular oxygen to form "active" oxygen which is then transferred to the drug The overall mechanism is shown in fig 1

Recent studies have established that a rate-limiting step in the overall oxidation of a drug by liver microsomal enzymes is the reduction of the cyto chrome P–450–drug complex Accordingly, species differences in the N demethylation of ethylmorphine by liver microsomes are more closely related to NADPH–cytochrome P–450 reductase activity than to the amount of cytochrome P–450, the magnitude of the "type I" spectral change, or the activity of NADPH-cytochrome c reductase (DAVIES *et al* 1969)

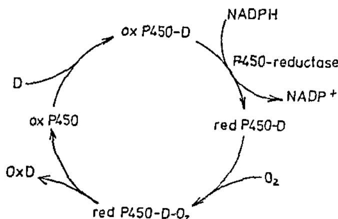


Fig 1 Oxidationofdrugs by liver microscomal enzymes Oxidized cytochrome P 450 (ox P 450) combines with drug (D) and the drug cytochrome complex is reduced by NADPH P-450 reductase The reduced cytochrome combines molecular oxygen (O) to give an 'active" oxygen complex Oxygen is then transferred to the drug to give oxidized drug (Ox D)

Individual differences in the plasma half-lives or plasma levels of lipid soluble drugs in man have been widely reported Often the magnitude of these differences far exceeds species differences in rates of metabolism For example, HAMMER *et al* (1966) reported a 36 fold variation in the steady-state plasma level of desmethylimipramine in a group of 11 patients receiving *the same* dose of the drug In addition VESELL & PAGE (1968a, 1968b & 1968c) have shown large individual differences in the plasma half lives of three drugs, phenylbutazone, antipyrine (phenazonum NFN) and dicoumarol For example in 28 subjects the phenylbutazone half-life varied from 1 2 to 7 3 days Similar variations were found for the other two drugs Individual differences in plasma half-lives are most often found for lipid soluble drugs which are not readily excreted by the kidney and which undergo extensive oxidation probably in the liver The plasma half-lives and therefore plasma levels of such drugs must depend to a large extent on the activity of the drug oxidizing enzymes of the liver *QUINN et al (1958) were able to relate species differences in plasma half-lives of hexobarbitone to differences in liver* enzyme activity

If the reduction of cytochrome P–450 is rate limiting in the oxidation of drugs then individual differences in rates of metabolism in man may be due to variations in P–450 reductase activities Given a common rate limiting step it ought to be possible to classify individuals according to their ability to oxidize drugs by measuring the rate of oxidation of one drug This classification could be of great use in selecting dosage regimens for patients on long term therapy

To examine this possibility we have measured the plasma half lives of a number of drugs in a group of healthy volunteers The drugs chosen are lipid soluble, not excreted unchanged in urine in significant amounts and metabolized mainly by oxidation Thus the plasma half lives of these drugs are probably determined by the rate at which they are oxidized in the liver

Methods

Eight healthy volunteers from our laboratory took part in the study The group consisted of 6 males and 2 females ranging in age from 23 to 39 years All subjects had not taken any drugs for at least one month before or during the study The drugs were administered as tablets, *antipyrine before meals* and phenylbutazone and oxyphenbutazone with meals. Determinations of half lives were spaced at intervals of at least three weeks.

Half lives were measured following two different dosage regimens. Firstly plasma concentrations were measured following a single oral dose of 10 mg/kg for each drug. In the second dosage regimen the drugs were given daily for 5 days (10 mg/kg for antipyrine and 5 mg/kg for phenylbutazone and oxyphenbutazone) at the end of

which the plasma level was monitored for at least one half life The latter dosage regimen was introduced to minimize the effect of absorption and distribution on decline of plasma levels of drugs

Plasma concentrations of antipyrine were measured according to the method of BRODIE & AXELROD (1950) and phenylbutazone and oxyphenbutazone concentrations were estimated as described by BURNS *et al* (1953) Mean half lives and the 95% confidence interval were obtained from the regression line calculated using a computer program

Results and Discussion

It was established that the plasma half-life of antipyrine in the same subjects did not differ following single and multiple doses (table 1) In addition antipyrine half-lives measured in the same subjects (S S T and A B in table 1) remained constant for periods of up to one year

Thus it would appear that plasma half-life of antipyrine can be easily and accurately measured in man and will remain constant in the same subject provided that no drug or environmental chemical causes an inhibition or induction of drug oxidizing enzymes

Antipyrine half-lives following single doses were measured in eight subjects and they ranged from 5 8 to 21 4 hours (table 2) Plasma half-lives of phenylbutazone were measured in the same group of volunteers following single and multiple doses Once more a range of values was observed However, from the results (table 2) it will be immediately obvious that plasma half-lives following the two dosage regimens were not the same in some subjects In W G, M E C, M O and C A, the plasma half-life was significantly shorter following five daily doses than after a single dose The

Table 1

Plasma half life of antipyrine (hours)

Subject	Single dose (10 mg/kg)	Five daily doses (10 mg/kg)
M O	9 6 ± 0 8	10 2 ± 1 5
S S T	10 6 ± 2.0 (9 1 69)*	10 4 ± 2 0
	10 4 ± 1 7 (3 7 69)	
	9 8 ± 2 3 (19 1 70)	
A B	18 0 ± 2 0 (16 1 69)	15 2 ± 2 3
	15 4 ± 2 5 (1 5 69)	
	18 3 ± 2 5 (7 7 69)	

* Date of measurement

Table 2

Subject	Antipyrine half life (hrs)	Phenylbutazone half life (hrs)	
	Single dose (10 mg/kg)	Single dose (10 mg/kg)	Five daily doses (5 mg/kg)
W G	5 8 ± 1 6	96 7 ± 6 9	47.5 ± 3 0
M.E.C.	9 8 ± 2 0	132 ± 22	64 4 ± 11 0
M O	9 6 ± 0 8	123 ± 17	64 9 ± 15 0
C.A.	10 0 ± 2 0	117 ± 13	73 0 ± 14 1
S.S T	10 3 ± 2 0	68 1 ± 11 1	69.5 ± 11 8
C.T D	11 7 ± 1 9	67.5 ± 13 0	69 0 ± 6 4
A.B	16 7 ± 2 5	102 ± 13	93 6 ± 11 4
D.S D	21 4 ± 2 4	132 ± 17	142 0 ± 9 0

interpretation of this change is difficult but important for the following reason From fig 2 it can be seen that antipyrine half-lives show a good correlation with multipledose phenylbutazone half-lives but little correlation with single-dose phenylbutazone half lives in this small group Thus one could, knowing a subject's antipyrine half life (single or multiple dose) predict his half life for phenylbutazone following five daily doses of the drug The same is obviously not true for phenylbutazone half-lives following single oral doses

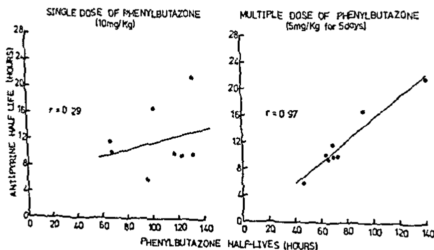


Fig 2 Correlation between antipyrine and phenylbutazone plasma half lives in the same subjects.

which the plasma level was monitored for at least one half life The latter dosage regimen was introduced to minimize the effect of absorption and distribution on decline of plasma levels of drugs

Plasma concentrations of antipyrine were measured according to the method of BRODIE & AXELROD (1950) and phenylbutazone and oxyphenbutazone concentrations were estimated as described by BURNS *et al* (1953) Mean half lives and the 95% confidence interval were obtained from the regression line calculated using a computer program

Results and Discussion

It was established that the plasma half-life of antipyrine in the same subjects did not differ following single and multiple doses (table 1) In addition antipyrine half-lives measured in the same subjects (S S T and A B in table 1) remained constant for periods of up to one year

Thus it would appear that plasma half-life of antipyrine can be easily and accurately measured in man and will remain constant in the same subject provided that no drug or environmental chemical causes an inhibition or induction of drug oxidizing enzymes

Antipyrine half-lives following single doses were measured in eight subjects and they ranged from 5 8 to 21 4 hours (table 2) Plasma half lives of phenylbutazone were measured in the same group of volunteers following single and multiple doses Once more a range of values was observed However, from the results (table 2) it will be immediately obvious that plasma half-lives following the two dosage regimens were not the same in some subjects In W G, M E C, M O and C A, the plasma half-life was significantly shorter following five daily doses than after a single dose The

Table 1

Plasma half life of antipyrine (hours)

Subject	Single dose (10 mg/kg)	Five daily doses (10 mg/kg)
M O	9 6 ± 0 8	10 2 ± 1.5
S S T	10 6 ± 2.0 (9 1 69)*	10 4 ± 2 0
	10 4 ± 1 7 (3 7 69)	
	9 8 ± 2.3 (19 1 70)	
A B	18 0 ± 2 0 (16 1 69)	15.2 ± 2 3
	15 4 ± 2.5 (1 5 69)	
	18 3 ± 2.5 (7 7 69)	

* Date of measurement

Another explanation of the change in plasma half-lives of phenylbutazone is that the drug when given acutely inhibits the drug oxidizing enzymes and therefore its own metabolism, but upon chronic administration the inhibition is overcome and if the drug is continued for a sufficient period of time stimulation of metabolism occurs It has been shown that many inducers have a similar biphasic effect on drug oxidation (SERRONE & FUJIMOTO 1961) In man it was observed (CHEN *et al* 1962) that when phenylbutazone and aminopyrine were given simultaneously there was an elevation of plasma levels of aminopyrine This was confirmed in rabbits and dogs and is in keeping with an initial inhibitory phase following a single dose of phenylbutazone Recent studies by CHO *et al* (1970) show that phenylbutazone causes a dose dependent non competetive inhibition of drug oxidation in rats pre treated with the drug The inhibition is observed for a period of 6 to 12 hours following dosing and at 24 hours induction of microsomal enzyme activity is observed

It would appear from data presently available that our multiple dose half lives for phenylbutazone were measured somewhere between the loss of inhibition of drug oxidation and the appearance of induction It is thus of great interest that for phenylbutazone, only plasma half lives determined following multiple doses correlate with antipyrine half lives It is possible that good correlation between half lives of different drugs in the same subjects will be found only when the drugs studied do not alter the activity of liver microsomal enzymes and therefore their own rate of metabolism

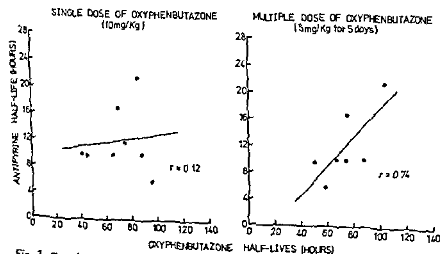


Fig. 3 Correlation between antipyrine and oxyphenbutazone plasma half lives in the same subject.

The obvious explanation of the change in phenylbutazone half lives was that in some subjects phenylbutazone induced the liver microsomal enzymes and thereby brought about an increase in its own rate of degradation It is known that pretreatment of rats with phenylbutazone increases the rate of oxidation of drugs such as hexobarbitone (CONNEY *et al* 1960) as well as the oxidation of phenylbutazone itself For several reasons it is not thought that the shortening of phenylbutazone half lives following five daily doses is due to induction

Firstly it was shown by CHEN *et al* (1962) that phenylbutazone accelerated aminopyrine metabolism in man only when it had been administered for a period of 4 days at a daily dose of approximately 10 mg/kg followed by six days at 5 mg/kg Our dosage is only 5 mg/kg for five days In addition it has been shown by VESELL & PAGE (1969) that degree of induction of antipyrine oxidation in man is related to a subject's ability to oxidize the drug prior to induction Thus subjects with long antipyrine half lives showed the greatest degree of induction with phenobarbitone If the same is true of phenylbutazone then we might accept subject D S D (antipyrine $t_{\frac{1}{2}}$ 22 hours) to be induced more readily than W G However whereas W G showed a 50 % shortening of phenylbutazone half life following multiple doses of the drug D S D did not show a significant change

The most convincing evidence that induction had not occurred following five daily doses is shown in table 3 Antipyrine half lives were measured in subjects before and following five daily doses of phenylbutazone It can be seen that in none of the subjects was there an accelerated decline of plasma antipyrine levels, indicating that phenylbutazone had not induced the oxidation of antipyrine in this study It would appear unlikely that phenylbutazone induced its own oxidation and not that of antipyrine In animal studies it was shown that in addition to increasing its own rate of metabolism phenylbutazone also increased the metabolism of hexobarbitone aminopyrine 3 4 benzpyrene (benzstigmimum NFN) and zoxazolamine

Table 3

Plasma half lives of antipyrine before and after daily administration of phenylbutazone (5 mg/kg) for 5 days

Subject	Antipyrine plasma half life	
	Before	After
W G	5 8 ± 1 6	10 0 ± 1 5
M O	9 6 ± 0 8	15 3 ± 2 0
C.T D	11 7 ± 1 9	15 1 ± 1 6

that for lipid soluble drugs individual differences in the rates of oxidation of drugs make a large contribution to differences in response Differences in response from this source at least could be largely eliminated if it were possible to measure a patient's ability to oxidize drugs With this knowledge the dose required to attain a desirable steady-state could be calculated Further studies are needed to determine whether the plasma half-life of antipyrine is a reliable functional test of liver microsomal enzymes

REFERENCES

Axelrod, J The enzymatic demethylation of ephedrine *J Pharmacol* 1955, 114, 430–438

Brodie, B B & J Axelrod The fate of antipyrine in man *J Pharmacol Exp Therap* 1950, 98, 97–103

Brodie, B B, J Axelrod, J R. Cooper, L. Gaudette, B N La Du, C. Mitoma & S Udenfriend Detoxication of drugs and other foreign compounds by liver microsomes *Science* 1955, 121, 603–604

Burns, J J., R. K. Rose, T Chenkin, A Goldman, A Schulert & B B Brodie. The physiological disposition of phenylbutazone (butazolidine) in man and a method for its estimation in biological material *J Pharmacol Exp Therap* 1953, 109, 346–354

Butler, T C. Termination of drug action by elimination of unchanged drug *Fedn Proc Fedn Am Soc Exp Biol* 1958, 17, 1158–1162.

Chen, W, P A Vrindten, P G Dayton & J J Burns Accelerated aminopyrine metabolism in human subjects pretreated with phenylbutazone. *Life Sci* 1962, 1, 35–42.

Cho, A. K., B J Hodshon & B B Brodie Effects of phenylbutazone on liver microsomal demethylase *Biochem Pharmacol* 1970, 19, 1817

Conney, A. H, C. Davison, R. Gastel & J J Burns Adaptive increases in drug metabolizing enzymes induced by phenobarbital and other drugs. *J Pharmacol Exp Therap* 1960, 130 1–8

Davies, D S, P L Gigon & J R. Gillette Species and sex differences in electron transport systems in liver microsomes and their relationship to ethylmorphine demethylation *Life Sci* 1969, 8, 85–91

Gillette, J R. Metabolism of Drugs and other Foreign Compounds by Enzymatic Mechanisms. In *Progress in Drug Research* Ed. E. Juchos Birkhauser Verlag, Basle, 1963

Gillette, J R *Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. Adv Pharmacol* 1966, 4, 219–261

Hammer, W., C. M Idestrom & F Sjöqvist Chemical control of antidepressant drug therapy In *Proc 1st Int Symposium on Antidepressant Drugs* 1966, No 122, Excerpta Medica Int. Congress Series.

Hammer, W, S Martens & F Sjoqvist *A comparative study of the metabolism of* desmethylimipramine, nortriptyline and oxyphenbutazone in man *Clin. Pharmacol Therap* 1969, 10, 44–49

Mason, H. S Mechanism of oxygen metabolism *Science* (N Y) 1957, 125, 1185–1188

Quinn, G P., J Axelrod & B B Brodie. Species, strain and sex differences in the metabolism of hexobarbitone, amidopyrine, antipyrine and aniline. *Biochem. Pharmacol.* 1958, 1, 152–159

Oxyphenbutazone, a metabolite of phenylbutazone, is also extensively oxidized in the liver and once more the rate of oxidation differs from one individual to another The plasma half life of oxyphenbutazone was measured in the group of volunteers using the two dosage regimens described for the previous drugs As with phenylbutazone half lives measured following five daily doses differed from those after a single dose of the drug But as is shown in fig 3 once more the correlation with antipyrine half lives is far better for the oxyphenbutazone half lives measured following multiple doses

Summary

There are large individual differences in the plasma half lives of lipid soluble drugs such as antipyrine, phenylbutazone and oxyphenbutazone Since these drugs are not excreted unchanged in urine in significant amounts it is reasonable to assume that these differences are due at least in part to differ ence in the activities of the liver enzymes oxidizing these drugs A recent study showed a four fold range in the rate of oxidation of ethylmorphine by human liver microsomal enzymes obtained from seven subjects (DAVIES & THORGEIRSSON unpublished results)

The plasma half life of antipyrine, which is constant in the same subjects over long periods of time, may be a good measure of an individual s ability to oxidize other drugs However, this requires that one can measure the plasma half life of drugs without the drugs themselves altering microsomal enzyme activity This appears to be difficult for two drugs studied namely phenylbutazone and oxyphenbutazone Following single doses the drugs appear to inhibit microsomal enzyme activity, but it returns to "normal" levels following dosing for five days Accordingly there was no correlation between antipyr
lives following s
daily doses half
with those for antipyrine in the same subjects HAMMER *et al* (1969) found a similar good correlation between steady state plasma levels of desmethylimi pramine and nortriptyline and the plasma half life of oxyphenbutazone fol lowing multiple doses of the latter drug It may be necessary in studies of correlations of rates of oxidation of different drugs in man to measure steady state plasma levels rather than half lives

Individual differences in response to standard doses of drugs present a considerable problem in clinical medicine, particularly when the therapeutic effect of the drug is not readily measured It is of some importance to con sider how clinicians can more easily tailor dosage regimens to suit the re quirement of individual patients It would appear from this and other studies

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Molecular Aspects of Drug Metabolism

By

Sten Orrenius

Abstract Drug oxidation is linked to a liver microsomal electron transport system consisting of at least two components – cytochrome P-450 and the NADPH cytochrome P-450 reductase Cytochrome P-450 is also involved in the hydroxylation of lipid soluble endogenous compounds, such as steroid hormones and fatty acids. Substrates capable of undergoing hydroxylation bind to cytochrome P-450 and the reduction of the cytochrome P-450-substrate complex so formed may well be the rate-limiting step in the over all hydroxylation process It is suggested that the competitive inhibition that various substrates exert on each other's hydroxylation is due to a competition for binding to a common cytochrome P-450 species in the liver microsomes

Key words Drug metabolism – hydroxylation – cytochrome P-450

It is now some 15 years ago that BRODIE and associates discovered the drug metabolizing activity of liver microsomes (BRODIE *et al* 1958) They showed that in the presence of NADPH and molecular oxygen, liver microsomes catalyze the hydroxylation of a variety of drugs and that, depending of the chemical nature of the drug, the reaction may lead to the oxidation of an aromatic ring or a hydrocarbon sidechain, an oxidative dealkylation or deamination, or the formation of a sulfoxide The system was similar to that first described 7 years earlier by MUELLER & MILLER (1948 & 1953) who had found that liver homogenates supplemented with NADH or NADPH in the presence of O_2 catalyzed the oxidative demethylation of the carcinogenic dye 4-dimethyl aminoazobenzene to 4-aminoazobenzene and formaldehyde Later it was found (HAYANO 1962) that microsomes from liver, adrenal cortex, testis, ovary, and placenta, catalyze an NADPH- and O_2-dependent hydroxylation of various steroids and that a steroid-hydroxylating system is also present in adrenal-cortex mitochondria Furthermore, the same system was found to be involved in the hydroxylation of aliphatic hydrocarbons and the ω-oxidation of fatty acids (ROBBINS 1961, WAKABAYASHI & SHIMAZONO 1961, DAS *et al* 1968)

Remmer, H , J Schenkman, R W Estabrook, H Sesame, J R. Gillette S Narasimhulu, D Y Cooper & O Rosenthal Drug interaction with hepatic microsomal cytochrome *Mol Pharmacol* 1966, **2**, 187–190

Serrone, D M & J M Fujimoto The diphasic effect of MPDC on the metabolism of hexobarbital *J Pharmacol Exp Therap* 1961, **133** 12–17

Vesell, E S & J G Page Genetic control of drug levels in man phenylbutazone. *Science* (N Y) 1968a, **159**, 1479–1480

Vesell, E S & J G Page Genetic control of drug levels in man antipyrine *Science* (N Y) 1968b, **161**, 72–73

Vesell, E S & J G Page Genetic control of dicoumarol levels in man *J Clin Invest* 1968c, **47**, 2657–2663

Vesell, E S & J G Page Genetic control of phenobarbitone induced shortening of plasma antipyrine half lives in man *J Clin Invest* 1969, **48** 2202–2209

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The hydroxylating enzyme system has been termed as a "mixed function oxidase" (MASON 1957) or "monooxygenase" (HAYAISHI 1962), since in the course of the reaction, one of the oxygen atoms of O_2 is incorporated into the compound undergoing hydroxylation, and the other into H_2O The process may be described by the general equation

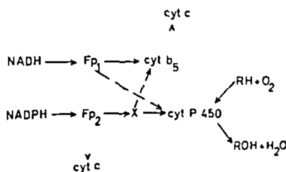


$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$$

where RH is the substrate and ROH the product of the hydroxylation reaction

It is now fairly well established that the microsomal hydroxylating system consists of at least two catalytic components (fig 1) a cytochrome called P-450 (OMURA & SATO 1964a and b), and the flavoprotein catalyzing the reduction of this cytochrome by NADPH, suitably termed NADPH cyto chrome P-450 reductase Cytochrome P-450 is characterized by a sensi tivity to carbon monoxide, which binds to the reduced form of the cyto chrome, giving rise to a characteristic absorption spectrum with a maximum at 450 nm (KLINGENBERG 1958, GARFINKEL 1958) The cytochrome is easily denatured and converted into a haemochromogen with a shift of the absorption maximum of its CO complex to 420 nm (OMURA & SATO 1964a and b) It is only recently that successful attempts have been re ported towards an extraction of cytochrome P-450 from microsomes in its native form, by a procedure that involves the use of glycerol and deoxycholate (LU *et al* 1969) The 'solubilized" cytochrome P-450 has been used for a reconstruction of the hydroxylating system, with NADPH-cytochrome P-450 reductase and a phospholipid fraction as additional com

ELECTRON TRANSPORT SYSTEMS
OF
LIVER MICROSOMES



1 Electron transport systems in liver microsomes

ponents A cytochrome P-450 has been purified from *Pseudomonas putida*, where it is involved in the hydroxylation of camphor (KATAGIRI *et al* 1968)

The flavoprotein NADPH-cytochrome P-450 reductase most probably is closely related to the enzyme known since 1950 as *NADPH-cytochrome c reductase* (HORECKER 1950), which in recent years has been purified and studied in great detail by several groups of investigators (LANG & NASON 1959, WILLIAMS & KAMIN 1962, PHILLIPS & LANGDON 1962) The purified enzyme has the interesting property of catalyzing an one electron transfer from NADPH to cytochrome *c*, its prostethic group FAD undergoing cyclic oxido-reductions between the half-reduced – free radical – and fully reduced states (KAMIN *et al* 1965) Cytochrome *c* appears to serve merely as an artificial electron acceptor for the enzyme, as does vitamin K_3 (menaquinone) and various redox dyes

Whether the flavoprotein interacts directly with cytochrome P-450 in the course of the hydroxylation process is not known, and there are indications that this may not be the case For example, neotetrazolium chloride can serve as an electron acceptor for NADPH oxidation in liver microsomes through a reaction that is insensitive to CO, i e, that does not involve cytochrome P-450, but shares the sensitivity of the hydroxylating system to SH reagents, in contrast to the NADPH-cytochrome *c* and menaquinone reductase reactions (ERNSTER & ORRENIUS 1965) Neotetrazolium chloride thus appears to interact with the system at a site intermediate between the flavoprotein and cytochrome P-450 The same holds true for the iron pyrophosphate catalyzed peroxidation of microsomal lipids (HOCHSTEIN & ERNSTER 1964, ERNSTER & NORDENBRAND 1967) The possibility has been considered that unsaturated microsomal lipids may serve as intermediate electron carriers between the NADPH oxidating flavoprotein and cytochrome P-450 (ERNSTER *et al* 1968)

There is also evidence for an interaction of the NADPH-linked hydroxylating system with the NADH-cytochrome *c* reductase present in liver microsomes, consisting of the flavoprotein NADH-cytochrome b_5 reductase and cytochrome b_5 (STRITTMATTER 1965) Such an interaction is indicated by the fact that, in liver microsomes, NADH is capable of reducing cytochrome P-450 and, conversely, NADPH can serve as a reducing agent for cytochrome b_5, although the rates of these "cross reactions" are lower than those of the NADPH-cytochrome P-450 reductase and NADH-cytochrome b_5 reductase, respectively (COOPER *et al* 1965, SATO *et al* 1965) Recent evidence suggests that these cross reactions are not due to a lack of specificity of the flavoproteins with respect to the reduced nicotinamide nucleotides, but rather to an interaction between the two electron transport chains in the flavin → cytochrome region, through intermediates that yet remain to be identified (ORRENIUS *et al* 1970, GNOSSPELIUS *et al* 1969)

The hydroxylating system of adrenal cortex mitochondria also contains cytochrome P-450, but differs from the microsomal system with respect to the flavoprotein The NADPH-oxidizing flavoprotein of adrenal cortex mitochondria does not react as such with cytochrome *c*, and its interaction with cytochrome P-450 is mediated by a non-heme iron protein, which also can serve as an electron transport link of the flavoprotein to cytochrome *c* (OMURA *et al* 1965, KIMURA & SUZUKI 1965 & 1967)

There is evidence that the reaction catalyzed by the hydroxylating system involves a binding of the substrate to cytochrome P 450 Such a binding has been inferred from spectral changes that occur when various substrates capable of undergoing hydroxylation are added to a suspension of microsomes (REMMER *et al* 1966, IMAI & SATO 1966, SCHENKMAN *et al* 1967) The spectral changes obtained with various substrates can be divided into two classes as illustrated in fig 2 a type I spectral change, characterized by a peak at about 385 nm and a trough at about 420 nm, and a type II spectral change, characterized by a trough in the 400 nm region and a peak in the 430 nm region of the difference spectrum of the microsomes That the spectral changes reflect binding of the substrate to cytochrome P-450 is shown by the fact that they are also observed with solubilized microsomal cytochrome P-450 (LU *et al* 1969) as well as with the cytochrome P-450 purified from *Pseudomonas putida* (GUNSALUS 1968) Of the two types of spectral changes, type I is generally believed to be due to a binding of the sub

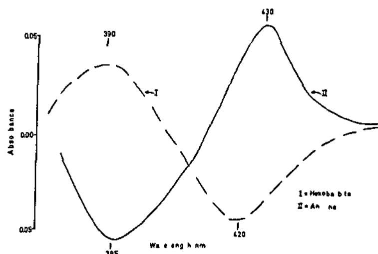


Fig 2 Spectral changes produced by the addition of hexobarbital (I) and aniline (II) to suspensions of rat liver microsomes Each cuvette contained in a volume of 2 9 ml 3 mg of microsomal protein and 50 mM tris Cl buffer, pH 7 5 Hexobarbital or aniline was added to the sample cuvette in a final concentration of 3 3 mM

Table 1

Concentrations of substances required for half maximal enzyme activities (K_m) and half maximal spectral changes (K_s)

Substrate	K_m µM	K_s µM	*Reference*
Hexobarbital	100	80	(Schenkman *et al* 1967)
Testosterone	10	8	(Orrenius *et al* 1970)
Laurate	6	4	(Orrenius *et al* 1970)

strate to the apoprotein of the cytochrome, whereas type II may at least partly be due to an interaction between the substrate and the heme prostethic group It is the type I spectral change that appears to be directly related to the hydroxylating activity Strong evidence for this relationship comes from the finding that the binding constants (K_s) of various substrates, defined as the concentration giving half maximal type I spectral change, closely parallel their Michaelis constants (K_m) with respect to the hydroxylating system, data in table 1 illustrate this correlation

The reaction sequence catalyzed by the microsomal hydroxylating system may thus be visualized to consist of the following steps (ESTABROOK *et al* 1968) 1 Reduction of the flavoprotein by NADPH, 2 Binding of substrate to the oxidized form of cytochrome P-450, 3 Reduction of the cytochrome P-450-substrate complex by the reduced flavoprotein, 4 Binding of O_2 to the reduced complex, 5 Dissociation of the oxidized cytochrome P-450 and the oxidized product

A problem of considerable interest concerns the substrate specificity of the hydroxylating system The overwhelming evidence now available for a direct interaction between the substrate and cytochrome P-450 in the course of the hydroxylating process makes it very unlikely that any further enzymes would be involved in the activation of the various substrates The question therefore arises as to whether one and the same cytochrome P-450 participates in the hydroxylation of the great variety of compounds that can serve as substrates of this enzyme system, or whether there exist different molecular species of cytochrome P-450, each specific for a given compound or group of compounds If the latter is the case, a further question is whether the various species of cytochrome P-450 are reduced through a common NADPH-cytochrome P-450 reductase or each one is reduced through a separate reductase

It has been shown that various substrates inhibit in a competitive manner the hydroxylation of each other when added in combination to liver microsomes in the presence of an excess of NADPH and oxygen (TEPHLY &

MANNERING 1968, ORRENIUS & THOR 1969, KUPFER & ORRENIUS 1970a) This effect which is illustrated in fig 3, and which has also been demonstrated with the isolated, perfused liver (VON BAHR *et al* 1970), is of considerable pharmacological interest, since it explains the well known clinical finding that drugs administered in combination may prolong each other's action and suggests, furthermore, that a similar interaction may occur between drugs and endogenous substrates of the hydroxylating system, e g steroid hormones The competitive relationship among various substrates with respect to their metabolism appears to eliminate the involve

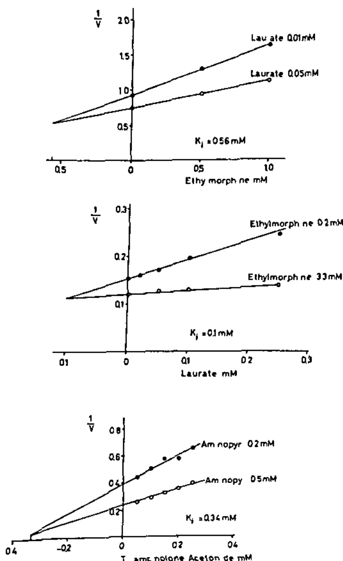


Fig 3 Competitive inhibition by various substances of each other's hydroxylation in rat liver microsomes Data compiled from ORRENIUS & THOR (1969) and KUPFER & ORRENIUS (1970)

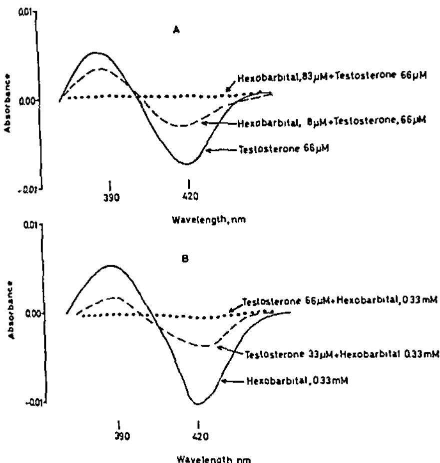


Fig. 4 Interaction of hexobarbital and testosterone in their binding to cytochrome P-450 of rat liver microsomes

A. Inhibition by hexobarbital of the magnitude of the type I spectral change produced by testosterone. Each cuvette contained, in a volume of 2.9 ml, 4 mg of microsomal protein in a 50 mM tris-Cl – 15 mM KCl medium, pH 7.5 ——— Testosterone (66 μM) was added in 20 μl ethanol to the sample cuvette, - - - - Hexobarbital (8 μM) was added to both the sample and reference cuvettes Testosterone (66 μM) was added in 20 μl ethanol to the sample cuvette, Hexobarbital (83 μM) was added to both the sample and the reference cuvettes. Testosterone (66 μM) was added in 20 μl ethanol to the sample cuvette When testosterone was absent the same amount of ethanol was added

B Inhibition by testosterone of the magnitude of the type I spectral change produced by hexobarbital. Eeach cuvette contained, in a volume of 2.9 ml, 4 mg of microsomal protein in a 50 mM tris-Cl 15 mM KCl medium, pH 7.5 ——— Hexobarbital (0.33 mM) was added in 20 μl ethanol to the sample cuvette, - - - - Testosterone (33 μM) was added to both the sample and the reference cuvettes. Hexobarbital (0.33 mM) was added to the sample cuvette, *Testosterone (66 μM)* was added in 20 μl ethanol to both the sample and the reference cuvettes. Hexobarbital (0.33 mM) was added to the sample cuvette. When testosterone was absent the same amount of ethanol was added.

ment of separate NADPH-cytochrome P-450 reductases in the hydroxylating system However, it does *not exclude* the existence of several species of cytochrome P-450 Clearly, if the NADPH-cytochrome P-450 reductase *portion* of the hydroxylating system is rate-limiting for the over-all process – *and there* are reasons to believe that so indeed is the case (GIGON *et al* 1969) – then different substrates will compete with each other's hydroxylation regardless of whether they utilize the same or different *species* of cytochrome P-450

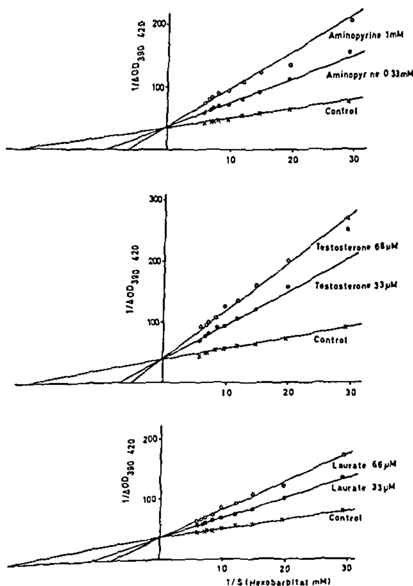


Fig 5 Competitive inhibition by aminopyrine, testosterone and laurate of the magnitude of the type I spectral change produced by the addition of hexobarbital to suspensions of rat liver microsomes For experimental conditions see Orrenius *et al* (1970)

A further approach to this problem may be based on investigation of the effect of various substrates of the hydroxylating system on their binding to cytochrome P-450 It is logical to assume that, if two substrates were bound to two different species of cytochrome P-450, they would give rise to an additive spectral change when both are added to microsomes If, on the other hand, they interact with the same species of cytochrome P-450, then saturation of the microsomes with respect to one substrate would eliminate the occurrence of a further spectral change upon the addition of the second substrate Experiments shown in fig 4, performed with hexobarbital (enhexymalum NFN) and testosterone as substrates, clearly demonstrate that the latter alternative is the case, indicating that these two substrates interact with the same species of cytochrome P-450 More over, as shown in fig. 5, combination of varying concentrations of the two substrates, as well as a number of other combinations of substrates, mutually influence their binding constants in a competitive fashion Thus, all of these substrates appear to bind to the same species of cytochrome P-450 in liver microsomes

Whereas liver microsomes readily catalyze the hydroxylation of various aromatic compounds (including many drugs), of certain steroids, as well as the oxidation of fatty acids, the hydroxylating systems of adrenal cortex microsomes and mitochondria seem to be "specialized" on steroids (HAYANO 1962) with a relatively weak capacity of the adrenal-cortex microsomal system to handle drugs, and virtually none to catalyze the oxidation of fatty acids (KUPFER & ORRENIUS 1970b) Kidney micro somes, on the other hand, which contain only very small amounts of cytochrome P-450, are remarkably active in catalyzing the oxidation of fatty acids (ICHIHARA *et al* 1969) while exhibiting little or no activity towards drugs and steroids (JAKOBSSON *et al* 1970) There is also a pronounced specialization among the hydroxylating systems of various tissues and organelles in regards to their way and ability to handle com pounds within a given group of substrates e g steroids (HAYANO 1962) It is difficult at the present time to rationalize these variations in specificity in relation to the concept of a single molecular species of cytochrome P-450 A possible explanation is that there exist various subspecies – isoenzymes – of cytochrome P-450, with different patterns of substrate specificity, and varying in relative abundance within the hydroxylating systems of various tissues and organelles Alternatively, it is conceivable that the same cytochrome P-450 molecule may assume different patterns of substrate specificity under influence of regulatory devices – e g allosteric effectors – present in the particular environments in the membrane structures which surround the cytochrome in the different tissues and organelles

There is now some evidence (JAKOBSSON & ORRENIUS 1970, unpublished observations) that compounds giving rise to a modified type II spectrum, and thus apparently binding to the cytochrome at a site different from its catalytic site, may promote the binding and metabolism of a substrate Preliminary experiments show, for example, that testosterone and laurate, both of which are *substrates of the hydroxylating system of liver microsomes*, giving rise to type I spectral changes and competing with each other in terms of both binding and metabolism, reveal an entirely different relationship in kidney microsomes Here, laurate is an active substrate, with a well-marked type I spectral shift and a high rate of metabolism, whereas testosterone gives rise to a modified type II spectral shift and is not metabolized at an appreciable rate On the other hand, testosterone in low concentrations enhances the rate of laurate oxidation in kidney microsomes with a simultaneous decrease in binding constant It thus appears that, whereas in liver microsomes both laurate and testosterone bind to the catalytic site of cytochrome P-450, in kidney microsomes laurate binds to the catalytic site of the cytochrome while testosterone binds to another locus of the enzyme which may have the function of a regulatory site

Acknowledgements

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REFERENCES

Bahr, C von, F Sjoqvist & S Orrenius The inhibitory effect of hydrocortisone and testosterone on the plasma disappearance of nortriptyline in the dog and the perfused rat liver *European J Pharmacol* 1970 9, 106–110

Brodie, B B, J R Gillette & B N La Du Enzymatic metabolism of drugs and other foreign compounds *Ann Rev Biochem* 1958, 27, 427–455

Cooper, D Y, S Narasimhulu, O Rosenthal & R W Estabrook Spectral and kinetic studies of microsomal pigments In *Oxidases and related redox systems* Ed T E King H S Mason and M Morrison New York, London and Sydney John Wiley & Sons, Inc, 1965 pp 838–855

Das, M L, S Orrenius & L. Ernster On the fatty acid and hydrocarbon hydroxylation in rat liver microsomes *Eur J Biochem* 1968, 4, 519–523

Ernster, L & K Nordenbrand Microsomal lipid peroxidation In *Methods in enzymology* Ed R. W Estabrook and M E. Pullman New York and London, Academic Press, 1967, Vol 10, pp 574–580

Ernster, L., K. Nordenbrand, S Orrenius & M L Das Microsomal lipid peroxidation *Hoppe-Seyler's Z Physiol Chem* 1968, 349, 1604–1605

Ernster, L. & S Orrenius Substrate-induced synthesis of the hydroxylating enzyme system of liver microsomes *Federation Proc* 1965, 24, 1190–1199

Estabrook, R. W., A. Hildebrandt, H. Remmer, J B Schenkman, O Rosenthal & D Y Cooper *The role of cytochrome P-450 in microsomal mixed function oxidation reactions*. In. *Biochemie des Sauerstoffs* Ed B Hess and Hj Staudinger Berlin, Heidelberg and New York, Springer-Verlag, 1968, pp 142–177

Garfinkel, D Studies on pig liver microsomes I Enzymic and pigment composition of different microsomal fractions *Arch Biochem. Biophys* 1958, **77**, 493–509

Gigon, P L., T E. Gram & J R. Gillette Studies on the rate of reduction of hepatic microsomal cytochrome P-450 by reduced nicotinamide dinucleotide phosphate effect of drug substrates *Mol Pharmacol* 1969, **5**, 109–122

Gnosspelius, Y, *H Thor & S Orrenius* A comparative study on the effects of phenobarbital and 3,4-benzpyrene on the hydroxylating enzyme system of rat liver microsomes *Chem Biol Interactions* 1969, **1** 125–137

Gunsalus, I. C. A soluble methylene hydroxylase system structure and role of cytochrome P-450 and iron sulfur protein components *Hoppe-Seyler's Z Physiol Chem* 1968, **349**, 1610–1613

Hayaishi, O History and scope In *Oxygenases* Ed O Hayaishi New York and London, Academic Press, 1962, pp 1–29

Hayano, M Oxygenases in lipid and steroid metabolism In *Oxygenases* Ed *O Hayaishi New York and London, Academic Press*, 1962, pp 181–240

Horecker, B L. Triphosphopyridine nucleotide-cytochrome c reductase in liver *J Biol Chem* 1950, **183**, 593–605

Hochstein, P & L. Ernster Microsomal peroxidation of lipids and its possible role in cellular injury In *Cellular injury* Ed. A V S de Reuck and J Knight London *J & A. Churchill Ltd., 1964* pp *123–134*

Ichihara, K, E. Kusumose & M Kusumose Some properties and distribution of the ω-hydroxylation system of medium chain fatty acids *Biochim Biophys Acta* 1969, **176**, 704–712

Imai, Y & R. Sato Substrate interaction with hydroxylase system in liver microsomes. *Biochem Biophys Res Commun* 1966, **22** 620–626

Jakobsson S, H Thor & S Orrenius Fatty acid inducible cytochrome P-454 of rat kidney cortex microsomes *Biochem Biophys Res Commun* 1970, **39**, 1073–1080

Kamin, H., *B S S Masters, Q H Gibson & C H Williams* Microsomal TPNH-cytochrome c reductase. *Federation Proc* 1965, **24**, 1164–1171

Katagiri, M., B N Ganguli & I C Gunsalus A soluble cytochrome P-450 functional in methylene hydroxylation. *J Biol Chem* 1968, **243**, 3543–3546

Kimura, T & K. Suzuki Enzymatic reduction of non heme iron protein (adrenodoxin) by reduced nicotinamide adenine dinucleotide phosphate. *Biochem Biophys Res. Commun* 1965, **20**, 373–379

Kimura, T & K Suzuki Components of the electron transport system in adrenal steroid hydroxylase and purification of a non heme iron protein (adrenodoxin) *J Biol Chem* 1967 **242**, 485–491

Klingenberg, M Pigments of rat liver microsomes. *Arch Biochem Biophys* 1958, **75**, 376–386

Kupfer D & S Orrenius Characteristics of guinea pig liver and adrenal monooxygenase systems. *Mol Pharmacol* 1970a, **6**, 221–230

Kupfer, D & S. Orrenius Interaction of drugs, steroids and fatty acids with microsomal cytochrome P-450 *Eur J Biochem.* 1970b, **14**, 317–322.

Lang, C A. & A. Nason A triphosphopyridine nucleotide-cytochrome c reductase from heart muscle. *J Biol. Chem* 1959, **234**, 1874–1877

Lu, A Y H, K W Junk & M J Coon Resolution of the cytochrome P-450 containing ω hydroxylation system of liver microsomes into three components. *J Biol Chem* 1969, **244**, 3714–3721

Mason, H S Mechanisms of oxygen metabolism *Adv Enzymol* 1957, **19**, 79

Mueller, G C & J A Miller The metabolism of 4 dimethylaminoazobenzene by rat liver homogenates. *J Biol Chem* 1948, **176**, 535–544

Mueller, G C. & J A Miller The metabolism of methylated aminoazo dyes. II Oxidative demethylation by rat liver homogenates *J Biol Chem* 1953, **202**, 579–587

Omura, T & R Sato The carbon monoxide binding pigment of liver microsomes. I Evidence for its hemoprotein nature *J Biol Chem* 1964a, **239**, 2370–2378

Omura, T & R Sato The carbon monoxide binding pigment of liver microsomes. II Solubiliaztion, purification and properties *J Biol Chem* 1964b **239**, 2379–2385

Omura, T, R Sato, D Y Cooper, O Rosenthal & R W Estabrook Function of cytochrome P 450 of microsomes *Federation Proc* 1965, **24**, 1181–1189

Orrenius, S & H Thor Fatty acid interaction with the hydroxylating enzyme system of rat liver microsomes *Eur J Biochem* 1969, **9**, 415–418

Orrenius, S, A Berg & L. Ernster Effects of trypsin on the electron transport systems of liver microsomes *Eur J Biochem* 1969, **11**, 193–200

Orrenius, S, D Kupfer & L Ernster Substrate binding to cytochrome P 450 of liver and adrenal microsomes *FEBS Letters*, 1970, **6**, 249–252

Phillips, A H & R G Langdon Hepatic triphosphopyridine nucleotide cytochrome *c* reductase isolation, characterization, and kinetic studies *J Biol Chem* 1962, **237** 2652–2660

Remmer, H, J B Schenkman, R W Estabrook, H Sasame, J R. Gillette, S Narasimhulu, C Y Cooper & O Rosenthal Drug interaction with hepatic microsomal cytochrome *Mol Pharmacol* 1966, **2**, 187–190

Robbins, K C. Enzymatic omega oxidation of fatty acids *Federation Proc* 1961, **20**, 272

Sato, R, T Omura & H Nishibayashi Carbon monoxide binding hemoprotein and NADPH specific flavoprotein in liver microsomes and their roles in microsomal electron transfer In *Oxidases and related redox systems* Ed T E King H S Mason and M Morrison New York, London and Sydney, John Wiley & Sons, Inc, 1965, pp 861–875

Schenkman, J B, H Remmer & R W Estabrook Spectral studies of drug interaction with hepatic microsomal cytochrome *Mol Pharmacol* 1967, **3**, 113–123

Strittmatter, P Protein and coenzyme interactions in the NADH cytochrome b_5 reductase system *Federation Proc* 1965, **24**, 1156–1163

Tephly, T R & G J Mannering Inhibition of drug metabolism V Inhibition of drug metabolism by steroids *Mol Pharmacol* 1968, **4**, 10–14

Wakabayashi, K & N Shimazono Studies *in vitro* on the mechanism of ω oxidation of fatty acids *Biochim Biophys Acta* 1961 **48** 615–617

Williams, C H & H Kamin Microsomal triphosphopyridine nucleotide cytochrome *c* reductase of liver *J Biol Chem* 1962, **237**, 587–595

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Kinetics of Drug Action in Man

By

Gerhard Levy

One of the major reasons for determining the time course of drug concentrations in body fluids, and for studying the factors affecting drug absorption, metabolism and excretion, is the realization that the intensity of many pharmacologic effects at a given time is related in some manner to the amount of drug distributed in the body at that time While this relationship can be very complicated, it has been possible to describe effectively the time course of many pharmacologic effects in man on the basis of some relatively simple pharmacokinetic relationships (LEVY 1966, 1967, 1970a & 1970b, LEVY *et al* 1969 & 1970, NAGASHIMA *et al* 1969, O'REILLY & LEVY 1970a & b) Some of the results of these studies will be described here

Let it be assumed that a drug is pharmacologically active as such and that its biotransformation products are inactive If drug elimination is an apparent first-order process, the amount of drug (A) remaining in the body at time t after intravenous injection of dose A_0 may be determined from the relationship

$$\log A = \log A_0 - \frac{k}{2.3} t \quad (1)$$

where k is the apparent first-order rate constant for drug elimination The intensity of many pharmacologic effects (E) is essentially linearly related to the logarithm of the amount of drug in the body (log A) Thus,

$$E = m \log A + e \quad (2)$$

where e is a negative value, representing the intercept on the E axis of the straight line which relates E to log A This approximately linear relationship, with a slope equal to m, usually holds in the clinically significant range Substitution from equation 1 in equation 2 and rearrangement yields

$$E = E_0 - \frac{km}{2.3} t \quad (3)$$

showing that the initial effect (E_o) declines at a constant rate (R) where

$$R = \frac{km}{2.3} \tag{4}$$

while the amount of drug in the body declines exponentially

If A in equation 1 is defined as the minimum effective dose (A_{min}), then t represents the duration of action of the drug Rearrangement of equation 1 yields

$$t = \frac{2.3}{k} (\log A_o) - \frac{2.3}{k} (\log A_{min}) \tag{5}$$

This equation predicts that a plot of duration of action versus the logarithm of dose should be linear irrespective of the pharmacologic end point used and that the elimination rate constant k should be calculable from the slope of the line The application of the simple pharmacokinetic relationships represented by equations 1 to 5 to clinical pharmacologic problems will be illustrated by means of several examples in the following paragraphs

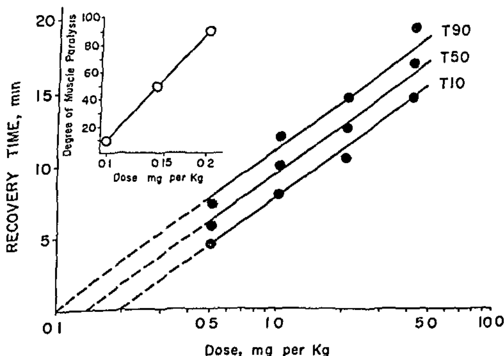


Fig 1 Relationship between intravenous dose of succinylcholine chloride and duration of various degrees of neuromuscular inhibition in human subjects T10 T50 and T90 are the times required to recover 10 50 and 90 % of normal muscle contraction force The data are from WALTS & DILLON (1967) and represent average values from 13–16 subjects for each dose Inset intensity of effect vs log dose, based on dose values obtained by extrapolation of duration of effect vs log dose plots (from LEVY 1967)

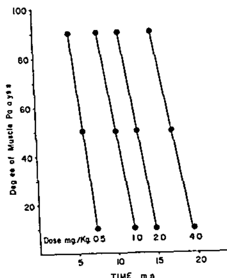


Fig. 2 Degree of muscle paralysis as a function of time after intravenous administration of 0.5 to 4.0 mg/kg succinylcholine chloride to groups of 13-15 human subjects Data from WALTS & DILLON (1967) (graph from LEVY 1967)

The duration of the neuromuscular blocking effect of succinylcholine (suxamethonum NFN) in man as a function of dose is shown in fig 1 The linear relationship is as predicted by equation 4 and yields an elimination rate constant of 0.20 min⁻¹ Extrapolation of the respective lines to $t = 0$ pro-

Table 1

Pharmacokinetic analysis of neuromuscular blocking effect of succinylcholine in nine infants*

Patient	Age days	Duration (t) min	Rate of decline (R) % min⁻¹	tR, %
11	28	5	40	200
10	24	6	40	240
14	37	6	40	240
15	38	6	40	240
7	10	7	20(27)**	140(189)**
4	3	9	27(30)	243(270)
2	1	12	27(30)	324(360)
5	10	14	16(17)	224(238)
8	20	26	9	234

* From LEVY (1970a) based on data from WALTS & DILLON (1969)
** Values in parentheses calculated by alternate methods as described in LEVY (1970a)

Table 2.

Neuromuscular blocking effect of suxamethonium in three cities

City	Dose	Recovery time (min)			Source of data	Rate of decline of effect, % min^{-1}	Rt* %
		10 %	50 %	90 %			
London	1 mg/kg	6 3 ± 2 15		9 1 ± 2 9	KATZ *et al* (1969)	28 6	180
New York	1 mg/kg	10 2 ± 3 2		14 6 ± 3 6	KATZ *et al* (1969)	18 2	186
Los Angeles	40 mg/m^2	7 0 ± 1 3	8 5 ± 1 6	10 0 ± 2 4	WALTS & DILLON (1969)	26 7	187

* Product of 10 % recovery time (t) and rate of decline of effect (R)
From LEVY (1970b), based on data from KATZ *et al* (1969)

vides A_{min} values for the three intensities of effect The E versus log A plot thus obtained is shown in the inset of fig 1 The relationship is linear and permits determination of m Consistent with equation 3, the neuromuscular blocking effect of succinylcholine declines at a constant rate independent of dose (fig 2) From the slopes of these data and from the previously obtained value of m it is possible to get another estimate of k. The value thus obtained (0 18 min $^{-1}$) is in very good agreement with the estimate derived from the duration of effect data in fig 1

Substitution of equation 4 in equation 5 and rearrangement yields

$$tR = m(\log A_0 - \log A_{min}) \qquad (6)$$

This equation shows that the product of duration (t) and rate of decline of effect (R) will remain constant as long as m, A_0 and A_{min} are the same. This makes it possible to determine if an unusually prolonged (or short) duration of action of a drug is due to unusually slow (or rapid) elimination It can be seen in table 1, for example, that the unusually protracted effect of succinyl-

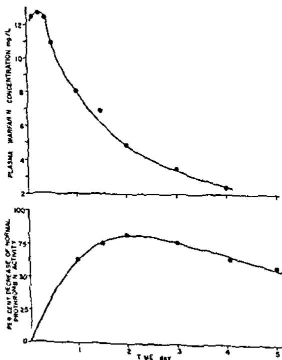


Fig. 3 Plasma warfarin concentration and depression of prothrombin complex activity as a function of time after oral administration of 1.5 mg warfarin sodium per kilogram body weight. Average of 5 normal subjects (from NAGASHIMA *et al* 1969)

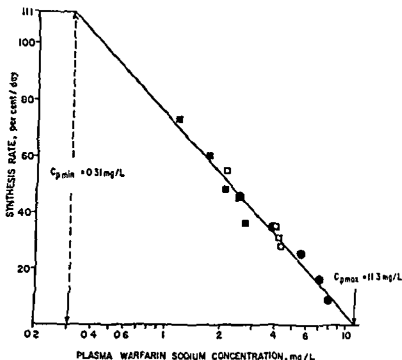


Fig 4 Synthesis rate of prothrombin complex activity as a function of plasma warfarin concentration, based on the averaged data from 6 normal subjects ("N average") Warfarin sodium dosing schedules ●, a single oral dose of 1.5 mg per kilogram, ■, daily oral doses of 10 mg for 5 days, □, daily oral doses of 15 mg for 4 days, $C_{p\,min}$, the apparent minimum effective plasma warfarin sodium concentration $C_{p\,max}$, the concentration of warfarin sodium in the plasma which apparently suppresses totally the synthesis of prothrombin complex activity (from NAGASHIMA *et al* 1969)

choline in two infants (No 5 and 8) is due to a low k, since the Rt values of these infants are normal Similarly, it can be shown that the difference in the response to succinylcholine in New York patients as compared to groups of patients in London and Los Angeles is clearly due to a lower elimination rate constant (k) in the New York group (table 2) Since the Rt values were similar in all three groups, it may be concluded that they did not differ with respect to m and A_{min}

A somewhat more complicated problem was the elucidation of the relationship between the anticoagulant effect of warfarin and bishydroxycoumarin (dicumarolum NFN) and the concentrations of these drugs in the plasma The maximum prothrombinopenic effect of warfarin occurs about two days later than the peak plasma levels (fig 3) However, prothrombin complex activity is a function of synthesis as well as degradation rate of clotting factors The coumarin anticoagulants only affect synthesis rate of factors II, VII, IX and X, and not degradation Thus, the "real" effect of these drugs is inhibition

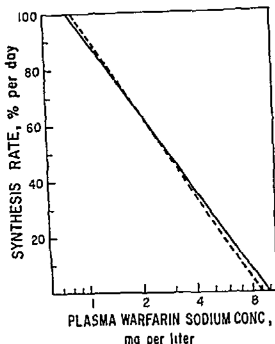


Fig. 5 Average relationship between synthesis rate of prothrombin complex activity and plasma warfarin concentration in control experiments (——) and during hepta barbital administration (– – – –) in 5 normal human subjects (from LEVY *et al* 1970)

of synthesis rate, and any correlation with plasma concentrations must be based on this effect rather than on the degree of inhibition of prothrombin complex activity Such data treatment (NAGASHIMA *et al* 1969) does indeed result in an excellent correlation between effect (expressed as one over-all process of synthesis of prothrombin complex activity) and plasma warfarin concentration (fig 4) Moreover, this method of pharmacokinetic analysis shows that enzyme inducers such as heptabarbital affect only the biotrans formation of the anticoagulant and not its distribution and affinity to the pharmacologic receptors (LEVY *et al* 1970) Thus, while the biologic half life of warfarin was decreased significantly by pretreatment with heptabarbital (LEVY *et al* 1970), the relationship between effect and plasma warfarin concentration remained unchanged (fig. 5) Similar results were obtained with bishydroxycoumarin (O REILLY & LEVY 1970a)

While the pharmacokinetic analysis of drug effects in man is only in its beginning it is already evident that such analysis is indeed often feasible, that it can be useful in elucidating the cause of unusual response to drugs, and that it can help in the design of safe and effective dosage regimens

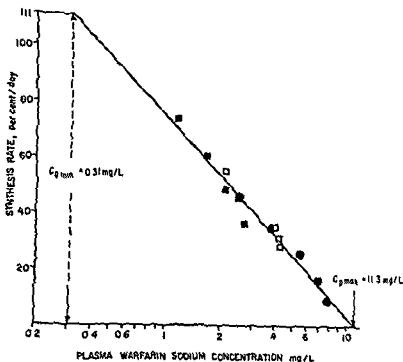


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Multiple Dose Kinetics and Drug Dosage in Patients with Kidney Disease

By

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This report deals with drugs which follow first order kinetics (Dettli & Spring 1968) and are eliminated partly by extrarenal processes (e g metabolism) and partly by the kidneys It follows that the total fraction of the amount of drug in the organism eliminated per hour (k) is the sum of the fraction removed by extrarenal elimination (k_m) and of the fraction eliminated by the kidneys, (k_r) (Dettli 1970a, c & d)

$$k = k_m + k_r \, (h^{-1}) \quad (1)$$

k, k_m and k_r are called the "overall", the extrarenal, and the renal elimination rate-constant, respectively It is assumed that the reduction of the overall elimination rate in patients with kidney disease is entirely due to a reduction of the renal elimination rate while the extrarenal elimination rate remains unaltered

When such a drug is administered repeatedly the drug level in the organism will eventually reach a steady state (Dettli 1970a & b) after a certain number of doses (see fig 1 and 2) Since this process of drug accumulation depends on the elimination rate (Dettli 1970a) (see below), the dosage schedule in patients with impaired drug elimination must be modified in order to avoid an increased risk of toxicity Our starting point for this purpose is the following basic postulate *"The dosage schedule must be modified in such a way that the drug level resulting in patients with kidney disease is the same and is reached after a similar time interval as in normal patients* (In this context the term "normal" applies to patients with normal renal function In our laboratory, a creatinine clearance of 100 ml/min is considered the normal mean value) It becomes evident that this problem can only be solved when the following questions are answered quantitatively

a. How is drug accumulation related to drug dosage and elimination rate?

b How is the drug s elimination rate related to the degree of renal impairment?

Most recently it has been possible to relate pharmacologic effects even to drug levels in specific compartments in multi-compartment systems (LEVY *et al* 1969, GIBALDI *et al* 1971)

REFERENCES

Gibaldi, M , G Levy, W L Hayton *Clin Pharmacol Therap* 1971, in press
Katz, R L , J Norman, R F Seed & L Conrad *Brit J Anesth* 1969, **41**, 1041
Levy, G *Clin Pharmacol Therap* 1966, **7**, 362
Levy, G *J Pharm Sci* 1967, **56**, 1687
Levy, G *Anesthesiology*, 1970a, **32**, 551
Levy, G *Brit J Anaesth* 1970b, **42**, 979
Levy, G , M Gibaldi & W J Jusko *J Pharm Sci* 1969, **58**, 422
Levy, G , R A O Reilly, P M Aggeler & G M Keech *Clin Pharmacol Therap* 1970, **11**, 372
Nagashima, R , R. A O Reilly & G Levy *Clin Pharmacol Therap* 1969, **10**, 22
O Reilly, R A & G Levy *Clin Pharmacol Therap* 1970a **11**, 378
O Reilly, R A & G Levy *J Pharm Sci* 1970b, **59**, 1258
Walts, L F & J B Dillon *Anesthesiology* 1967, **28**, 372
Walts, L F & J B Dillon *Anesthesiology* 1969, **31**, 35

dosage interval (τ) and inversely proportional to the drug's overall elimination rate (k) (DETTLI 1970a, b & c). Thus, if the average overall elimination rate in "normal" patients is denoted by k_n we may write

$$m_\infty = \frac{D/\tau}{k} \tag{2}$$

If the symbols relating to a patient with renal impairment are denoted by circumflex marks (^) the analogous relationship for such a patient will read

$$\hat{m}_\infty = \frac{\hat{D}/\tau}{\hat{k}} \tag{3}$$

Since our basic postulate of identical steady state levels means $\hat{m}_\infty = m_\infty$, it follows from eq. 2 and 3 that

$$\hat{D} = D \cdot \frac{\hat{k}}{k_n} \tag{4}$$

b. *The loading dose.* The time-course of the accumulation process during continuous administration of a drug follows the equation (DETTLI 1970a & b)

$$m(t) = m_\infty \cdot (1 - e^{-kt}) \tag{5}$$

Eq. 5 expresses the following statement: "The lower the drug's elimination rate the slower is the time-course of accumulation and vice versa (see fig. 1). Hence in drugs with rapid elimination (e.g. penicillin), m_∞ is practically reached after a few hours only. Therefore, the administration of a loading dose is not necessary. However the elimination rate of the same drug may be decreased to such an extent in patients with kidney impairment that it may take several days until the steady state level, $\hat{m}_\infty$, is reached. In order to reach $\hat{m}_\infty$ immediately a loading dose, $\hat{D}^*$, must be administered. This loading dose must be equal to the amount of drug present in the organism after reaching the steady state level, or in mathematical terms $\hat{D}^* = \hat{m}_\infty$. It follows from eq. 3

$$\hat{D}^* = \hat{m}_\infty = \frac{\hat{D}/\tau}{\hat{k}} \tag{6}$$

B. *Intermittent administration*

a. *The maintenance dose.* When the maintenance dose is administered intermittently at regular dosage intervals a steady state level will be reached as with continuous administration (DETTLI 1970a & b). However, as depicted schematically in fig. 2, the level will fluctuate between a maximum ($m_{0\infty}$)

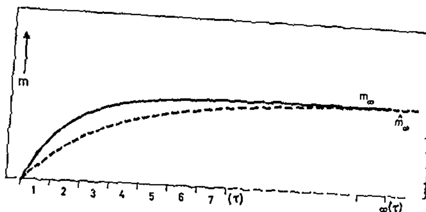


Fig 1 The accumulation of a drug during its continuous administration in a p with normal (uninterrupted curve) and in a patient with impaired kidney function ted curve) The drug level increases until a steady state is reached It was ass that the half life in the patient with kidney impairment is twice as long as i normal patient Therefore the dose administered was reduced according to eq a result identical steady state levels are obtained in both patients ($\hat{m}_\infty = m_\infty$) that the time course of drug accumulation is slower in the patient with k impairment

The dosage schedule as a function of the drug's elimination rate

A *Continuous drug administration*

a *The maintenance dose* The steady-state level reached in the organi (m_∞) is proportional to the maintenance dose (D) administered during

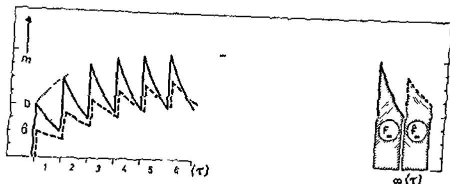


Fig 2 Intermittent administration Drug accumulation in a patient with normal (uninterrupted curve) and in a patient with impaired kidney function (dotted curve) In both patients the amount of drug in the organism (m or $\hat{m}$) increases after each dose until a steady state level is reached which fluctuates between a maximum at the beginning and a minimum at the end of the dosage intervalls It was assumed that the half life in the patient with kidney impairment is twice as long as in the normal" patient Therefore the dose was reduced according to eq 9 As a result one obtains in both patients identical areas below the curves ($F_\infty - \hat{F}_\infty$ The time course of accumulation is again slower in the patient with impaired kidney function

Since the half-life in patients with normal kidney function is known for a great number of drugs, k_n is easily obtained by means of eq 11

b *The individual rate constant of elimination in the patient with renal impairment* This would mean the determination of the half-life of the drug in each individual patient In practice this is unrealistic In order to solve this problem several authors (AZZOLINI *et al* 1970, CUTLER & ORME 1969, DETTLI & SPRING 1968, DETTLI 1970a,b,c & d, DOHERTY *et al* 1964, GINGELL & WATERWORTH 1968, JELLIFFE 1967, ORME & CUTLER 1969, REUBI & MUNGER 1968, REUBI & VORBURGER 1969) have tried to demonstrate a quantitative relationship between renal function tests and drug elimination rate In many drugs it may be derived from BRICKER *et al* s "intact nephron hypothesis" (1960) that the renal elimination rate, k_r, is proportional to the glomerular filtration rate, i e to the inulin clearance Unfortunately the inulin clearance is too complicated as a routine procedure In contrast, the endogenous creatinine clearance, V_{cr}, is becoming an accepted routine test of kidney function Therefore, it may be tentatively assumed that k_r is approximately proportional to V_{cr}, or in mathematical terms

$$k_r = a \cdot V_{cr} \tag{12}$$

Introducing eq 12 into eq 1 results in

$$k = k_m + a \cdot V_{cr} \tag{13}$$

According to eq 13 the relation between k and V_{cr} is described by a straight line (see fig. 3) Its point of intersection with the ordinate at $V_{cr} = 0$ corresponds to the extrarenal elimination rate (k_m) in the anuric patient. On the other hand, k_n, corresponds to the value of k in patients with a normal creatinine clearance (100 ml/min) In any patient with a creatinine clearance below 100 ml/min and above 0 ml/min the elimination rate, $\hat{k}$ can be estimated by means of eq 13 once the mean values of k_m and a in eq 13 are determined This requires the simultaneous measurement of the creatinine clearance and of the drug s *elimination rate in a representative* sample of patients with different degrees of kidney insufficiency A great number of such measurements have been published in the literature after the pioneer work of KUNIN (KUNIN & FINLAND 1959a & b, KUNIN *et al* 1959a & b) The typical example depicted in fig. 3 represents the current status of our investigation project with the experimental cephalosporin compound 36278-Ba* From these data the linear correlation between k and V_{cr} was calculated according to eq 13 using the method of the least squares of errors. The following equation was found

*) *Compound* 36278-Ba (7-cyanoacetyl aminocephalosporanic acid) was supplied by Ciba A. G., Basel (Switzerland)

at the beginning and a minimum (mτ ∞) at the end of the dosage intervals As shown elsewhere (DETTLI 1970c), the area below this curve, i e the time-concentration (or time-amount) integral (F_∞ or $\hat{F}_\infty$ in fig 2) is proportional to the dose and inversely proportional to the "overall" rate-constant of elimination, k Thus, in a "normal" patient we will have

$$F_\infty = \frac{D}{k_n} \tag{7}$$

and in the patient with kidney impairment we obtain

$$\hat{F}_\infty = \frac{\hat{D}}{\hat{k}} \tag{8}$$

When we wish to have the same areas below the curve in the patient with kidney impairment and in the "normal" patient, i e $\hat{F}_\infty = F_\infty$ – the following dosage equation results from eq 7 and 8

$$\hat{D} = D \frac{\hat{k}}{k_n} \tag{9}$$

It is noteworthy that the same equation can be used for continuous (eq 4) and for intermittent (eq 9) drug administration

b *The loading dose* The time-course of accumulation during intermittent drug administration may be described by substituting n τ for t in eq 5 (n = number of doses administered) It follows that the same considerations apply as in the case of continuous administration The correct loading dose is arrived at by multiplying the maintenance dose, $\hat{D}$, by the "dose ratio" $R^* = D^*/D$ according to KRUGER-THIEMER (1960) and DETTLI (1970a)

$$\hat{D}^* = \hat{D}\ R^* = \hat{D}\ \frac{1}{1 - e^{-\hat{k}\,\tau}} \tag{10}$$

The elimination rase as a function of the endogenous creatinine clearance

It follows from the above equation that dosage schedules which fulfill our basic postulate can be calculated for any individual patient with kidney impairment provided the following information is available

a *The mean 'normal' rate-constant of elimination (k_n)* There exists a simple relationship between the rate constant of elimination, k, and the biological half-life, $t_{\frac{1}{2}}$ of a drug (*DETTLI 1970b*)

$$k = \frac{\ln 2}{t_{\frac{1}{2}}} \text{ or } t_{\frac{1}{2}} = \frac{\ln 2}{k} \approx \frac{0\ 7}{k} \tag{11}$$

Table 1

Rate-constants of drug elimination in anuric patients (k_m) and in patients with normal kidney function (k_n)

Drug	k_n	k_m
Penicillin G (DETTLI & SPRING, unpublished results, KUNIN & FINLAND 1959b)	1 4	0 03
Ampicillin (REUBI & VORBURGER 1969)	0 6	0 11
Methicillin (KUNIN 1967b)	1 4	0 17
Oxacillin (KUNIN 1967b)	1 4	0.35
Cephalothin (DETTLI & SPRING, unpublished results, KABINS & COHEN 1964, KUNIN & ATUK 1966, YAMASAKU *et al* 1970)	1 4	0 06
Cephaloridine (DETTLI 1970d, DETTLI & SPRING, unpublished results, KABINS & COHEN 1965, KUNIN & ATUK 1966, PRYOR *et al* 1967, YAMASAKU *et al* 1970)	0 4	0 03
Cephalexin (BAILEY *et al* 1970, YAMASAKU *et al* 1970)	0 7	0 03
Ciba 36278	0 7	0 03
Tetracycline (KUNIN *et al* 1959a)	0 08	0 008
Chlortetracycline (KUNIN *et al* 1959a)	0 12	0 08
Rolitetracycline (REUBI & MÜNGER 1968)	0 06	0 02
Doxycycline (FABRE *et al* 1967, MANNHART & DETTLI, unpublished results)	0 03	0.03
Chloramphenicol (KUNIN *et al* 1959b, LINDBERG *et al* 1966)	0.3	0.2
Thiamphenicol (AZZOLINI *et al* 1970)	0.26	0 02
Streptomycine (KUNIN & FINLAND 1959b)	0.27	0 01
Kanamycin (CUTLER & ORME 1969, JELLIFFE, personal communication, KUNIN & FINLAND 1959b, ORME & CUTLER 1969)	0.252	0 01
Vancomycin (KUNIN 1967b)	0 12	0 003
Gentamycin (CURTIS *et al* 1967, GINGELL & WATERWORTH 1968, KUNIN 1968)	0.3	0 02
Colistin (GOODWIN & FRIEDMAN 1968, KUNIN 1968, NAUMANN & ANWÄRTER 1964)	0.31	0 08
Polymyxin B (KUNIN 1967b)	0 16	0 02
Erythromycin (KUNIN & FINLAND 1959b)	0 5	0 13
Lincomycin (KUNIN 1967b)	0 15	0 06
Rifampicin (DETTLI & SPRING, unpublished results)	0 25	0 25
Sulfamethoxazole (DETTLI & SPRING, unpublished results)	0 07	0 07
Digoxin (DETTLI & SPRING unpublished results, DOHERTY *et al.* 1964, 1967, 1968 & 1969, EWY *et al* 1969 JELLIFFE 1967 KRAMER *et al.* 1970)	0 017	0 008
Methyldigoxin (KRAMER *et al.* 1970)	0 016	0 007
Digitoxin (KRAMER *et al* 1970)	0.004	0 003
Strophanthin G (KRAMER *et al*)	0 05	0 012
Peruvoside (KRAMER *et al.* 1969 & 1970, LAHRTZ & VAN ZWIETEN 1968)	0 01	0 012

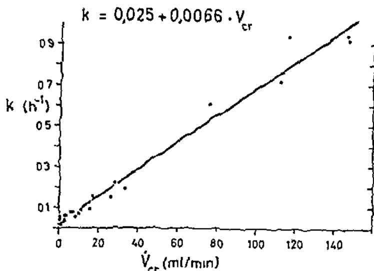


Fig 3 The elimination rate (k) of compound 36278 Ba as a function of the endogenous *creatinine clearance* (V_{cr}) in 26 patients with different degrees of renal impairment The correlation is significant at the level of $P < 0\,001$

$$k = 0\,025 + 0\,0066 \quad V_{cr} \tag{14}$$

From eq 14 one obtains the following characteristic values for this drug $k_n = 0\,685$ and $k_m = 0\,025$ per hour

According to eq 11 the corresponding half-lifes are $t_{\frac{1}{2},n} = 1{,}0$ hour in the "normal" patient and $t_{\frac{1}{2},m} = 27{,}7$ hours in the anuric patient

Based on the results published in the literature and on our own experiments it is possible at present to calculate k_n and k_m according to eq 13 and 11 for the 28 agents listed in table 1

A simplified method for determining dosage schedules

Using the values in table 1 one can calculate the numerical solution of eq 13 for these drugs The equation for the corrected maintenance dose is obtained by substituting $\hat{k}$ in eq 4 by eq 13

$$\hat{D} = D \quad \frac{k_m + a \quad V_{cr}}{k_n} \tag{15}$$

Eq 15 proved too complicated for clinical use Therefore, a graphical method was worked out which is being used at the University Hospital in Basel The method is based on the following Since the correlation between the elimination rate of the drug and the endogenous creatinine clearance is linear, the corresponding straight line is completely defined by two points These points are k_n and k_m With these values one can determine the individual value of $\hat{k}$ in a patient with kidney impairment according to the following rules

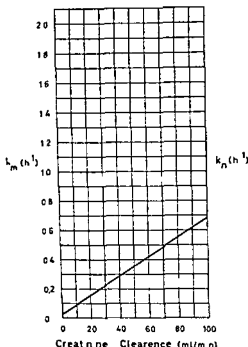


Fig. 4 Chart for the graphical determination of individual elimination constants ($\hat{k}$) in patients with impaired kidney function. Abscissa = endogenous creatinine clearance (V_{cr}) Ordinate = elimination constant (k) The right ordinate (k_n) is drawn at the creatinine clearance value considered normal (e g $V_{cr} = 100$ ml/min) the left ordinate (k_m) at $V_{cr} = 0$ ml/min The "dosage line" of compound 36278-Ba is shown as an example For further explanations see text

cides that the appropriate maintenance dose (assuming normal kidney function) would be 6000 mg of compound 36278 Ba per 24 hours by continuous intravenous infusion But he learns that the creatinine clearance of the patient is only 10 ml/min For this value the dosage line in fig 4 indicates an elimination rate contsant $\hat{\lambda} = 0.09$ per hour corresponding to a half life of 7 7 hours (eq 11) The maintenance dose $\hat{D}$ for this patient is obtained according to eq 4

$$\hat{D} = 6000 \frac{0.09}{0.69} = 783 \text{ mg} \cong 800 \text{ mg/24 hrs}$$

Introducing this value of $\hat{D}$ into eq 6 gives the correct loading dose

$$\hat{D}^{*} = \frac{783/24}{0.09} = 363 \text{ mg} \cong 400 \text{ mg}$$

a The physician has first to decide the following Assuming that his particular patient had normal kidney function, what would be the maintenance dose, D?

b k_n and k_m of the drug (table 1) are then plotted on a chart such as depicted in fig 4 k_n on the right ordinate (at $V_{cr} = 100$ ml/min) and k_m on the left ordinate (at $V_{cr} = 0$ ml/min) A straight line is then drawn between k_n and k_m This line is called the "dosage line" The point of intersection between the creatinine clearance of the patient and the "dosage line" of the drug represents the individual elimination constant, $\hat{k}$, of the patient

c The maintenance dose, $\hat{D}$, is now calculated by introducing these values of D, k_n and $\hat{k}$ into eq 4 or 9 The loading dose, $\hat{D}^*$, is calculated according to eq 6 or 10

The following example will illustrate the procedure Let us consider a hypothetical patient with a serious bacterial infection The physician de-

Table 2

Equations for the calculation of intermittent dosage schedules in patients with impaired kidney function In principle, the dosage schedule may be adapted by reducing the maintenance dose, D (left column) or by prolonging the dosage interval τ (right column) Eq 4 (or 9) which is valid for intermittent and continuous administration fulfills the postulate of identical time concentration integrals ($\hat{F}_\infty = F_\infty$) Eq 17 leads to identical minima ($\hat{m}_{\tau,\infty} = m_{\tau\,\infty}$) and eq 18 to identical maxima ($\hat{m}_{o\,\infty} = m_{o\,\infty}$) Eq 19 meets all of these postulates *When a drug is used in capsule or other similar* form the calculated dose D may be smaller than the content of one capsule In this case the dosage interval $\hat{\tau}$ *should be prolonged to the same extent as the cal* culated dose $\hat{D}$ is *increased* The correct loading dose, $\hat{D}^*$, for intermittent administration is calculated according to eq 10

Postulate	$\hat{D} < D$ & $\hat{\tau} = \tau$	$\hat{\tau} > \tau$ & $\hat{D} = D$
$\hat{F}_\infty = F_\infty$	$\hat{D} = D \frac{\hat{k}}{k_n}$ (4) (9)	
$\hat{m}_{\tau,\infty} = m_{\tau\,\infty}$	$\hat{D} = D \frac{e^{k\tau}(1 e^{\hat{k}\tau})}{e^{\hat{k}\tau}(1 e^{k\tau})}$ (17)	$\hat{\tau} = \tau \frac{k_n}{\hat{k}}$ (19)
$\hat{m}_{o,\infty} = m_{o\,\infty}$	$\hat{D} = D \frac{1 e^{\hat{k}\tau}}{1 e^{k\tau}}$ (18)	
$\hat{D}^*$	$\hat{D}^* = \frac{D}{1 e^{\hat{k}\tau}}$ (10)	$\hat{D}^* = \frac{D}{1 e^{\hat{k}\hat{\tau}}}$ (10)

KUNIN *et al* 1959a & b, KUNIN & ATUL 1966, KUNIN 1967a,b & 1968) In principle KUNIN uses a special modification of eq 19 (see table 2) In the anuric patient a full, normal dose is recommended as a loading dose Then the agent is administered as half that dose at dosage intervals corresponding to the mean half life of the drug in anuric patients (KUNIN 1967b) From a kinetic view point this is a sound procedure because, with $\tau = t_{\frac{1}{2}}$, eq 10 yields $D^*/D = 2\,0$ However, using this method, the time concentration integral ($\hat{F}_\infty$) in the severely uremic patient can be tremendously elevated even though the peak values are the same as in the normal patient Those side effects which depend on the concentration peak as well as on its duration are therefore more probable in the anuric patient. For this reason we prefer in most cases dosage schedules according to eq 9 or 17 and especially so in the case of bacteriostatic agents where a minimum inhibitory concentration should be maintained

Furthermore, KUNIN advocates the dosage schedule mentioned above for patients with a creatinine clearance between 0 and 10 ml/min Uremic patients whose creatinine clearance is in excess of 10 ml/min receive the same doses twice as frequently KUNIN clearly recognized that "exact dosage schedules are difficult to formulate in such individuals because of

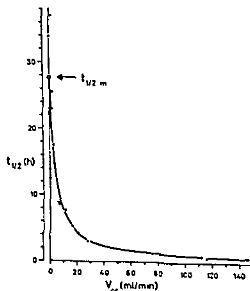


Fig. 5 The half life ($t_{\frac{1}{2}}$) of compound 36278-Ba as a function of the endogenous creatinine clearance (V_{cr}) in 26 patients. The half lives were calculated according to eq 11 from the elimination constants plotted in fig. 1 The hyperbolic curve is described by eq 16.

In the case of intermittent administration, e g 3 g every 12 hours, eq 9 leads to the following maintenance dose in this patient

$$\hat{D} = 3000 \; \frac{0\,09}{0\,69} = 390 \text{ mg} \cong 400 \text{ mg}$$

The corresponding loading dose is found according to eq 10

$$\hat{D}^* = \frac{390}{1 - e^{-0\,09\;12}} = 590 \text{ mg} \cong 600 \text{ mg}$$

Discussion

Among the many problems connected with drug dosage in renal failure the following points will be discussed

It should be emphasized that the data listed in table 1 are incomplete in many respects and may have to be changed as more experimental material accumulates The most unequivocal results are those of REUBI & MUNGER (1968) and REUBI & VORBURGER (1969), because the inulin clearance was used instead of the creatinine clearance Before these results can be used the creatinine clearance methods of the different laboratories must be standardized with the inulin clearance as a reference (HEALY 1968)

With continuous drug administration, eq 4 and 5 predict identical time-concentration curves in the "normal" patient and in the patient with kidney disease The problem is more complex with intermittent administration, because even theoretically it is impossible to fulfill the postulate of identical drug levels in its strict sense since $k_n > \hat{k}$ the slope of the concentration curve must be steeper in the normal patient than in the patient with kidney disease (see fig 2) If the dosage schedule is calculated according to eq 4 only the *areas* below the curves will be identical, whereas the maxima will be lower and the minima will be higher in the patient with kidney disease However, the therapist may possibly prefer a 'Kruger-Thiemer type" dosage schedule (KRUGER-THIEMER 1960) with identical minimum values (e g when a reversibly acting drug such as a bacteriostatic agent is used) or one with identical peak values (e g when an irreversibly acting drug such as a bactericidal agent is used) Furthermore, the dosage schedule may be modified with different results by reducing the maintenance dose or by prolonging the dosage interval, or both Consequently, there are at least six different ways to calculate the individual dosage schedule for one and the same patient The corresponding equations have been published elsewhere A synopsis is given in table 2

Any new dosage theory for patients with renal failure must be discussed in the light of KUNIN s work in this field (KUNIN & FINLAND 1959a & b

KUNIN *et al* 1959a & b, KUNIN & ATUL 1966, KUNIN 1967a b & 1968) In principle KUNIN uses a special modification of eq 19 (see table 2) In the anuric patient a full, normal dose is recommended as a loading dose Then the agent is administered as half that dose at dosage intervals corresponding to the mean half life of the drug in anuric patients (KUNIN 1967b) From a kinetic view point this is a sound procedure because, with $\tau = t_{\frac{1}{2}}$, eq 10 yields $D^*/D = 2.0$ However, using this method, the time concentration integral ($\bar{F}_\infty$) in the severely uremic patient can be tremendously elevated even though the peak values are the same as in the normal patient Those side effects which depend on the concentration peak as well as on its duration are therefore more probable in the anuric patient For this reason we prefer in most cases dosage schedules according to eq 9 or 17 and especially so in the case of bacteriostatic agents where a minimum inhibitory concentration should be maintained

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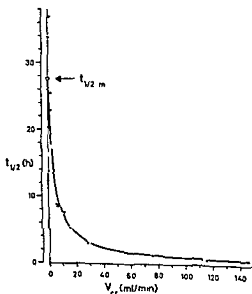


Fig. 5 The half life ($t_{\frac{1}{2}}$) of compound 36278-Ba as a function of the endogenous creatinine clearance (V_{cr}) in 26 patients. The half lives were calculated according to eq 11 from the elimination constants plotted in fig. 1 The hyperbolic curve is described by eq 16

the nature of the curve relating half-life to renal function " (KUNIN 1967b) This curve is easily obtained by introducing eq 13 into eq 11

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} = \frac{\ln 2}{k_m + a \quad V_{cr}} \tag{16}$$

As can be seen from eq 16 the curve relating half-life to creatinine clearance is a hyperbola

Fig 5 illustrates this point the same results are depicted as in fig 3, the difference being that the ordinate represents the half life rather than the elimination constant of the drug It is evident from fig 5 that the most dramatic increase in half-life occurs at clearance values between 10 and 0 ml/min It becomes clear that it is hardly possible to derive individualized dosage schedules for these patients using the half-life as a measure of elimination Therefore, in our dosage theory the elimination constant is used rather than the half-life From this discussion it becomes clear that the dosage theory presented is not contrary to but rather supplements KUNIN's dosage recommendations

It should be realized that the drug's metabolites are not considered in our theory Therefore such metabolites may accumulate tremendously (KUNIN *et al* 1959b) even though the dosage schedule of the drug itself has been adapted to the patient's renal capacity The physician should be aware of the possible side effects of these metabolites

Recently we found some preliminary evidence that the rate of metabolic drug transformation (k_m) may be reduced in some of the severely uremic patients and in some patients with liver impairment

When the drug level m (mg) is divided by the drug s distribution volume (l) our equations predict the time course of the drug's plasma concentration (mg/l) This means that these equations constitute a quantitative hypothesis which can *be tested by comparing the plasma concentrations* predicted by the theory with the experimentally determined values Such experiments are under way in our clinic

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REFERENCES

Azzolini, F, A Gazzaniga & E Lodola Thiamphenicol excretion in subjects with renal insufficiency *Int J clin Pharmacol* 1970 4, 303–308

Bricker, N S, P A F Morrin & S W Kime The pathologic physilogy of chronic Bright s disease *Amer J Med* 1960 28, 77–98

Bailey, R. R, P E Gower & C H. Dash Effects of impairment of renal function and hemodialysis on serum and urine levels of cephalexin. *Postgrad med J* 1970, 46, Suppl 60–64

Curtis, J R., S J McDonald & J H Weston Parenteral administration of gentamycin in renal failure patients undergoing intermittent haemodialysis. *Brit med J* 1967, I, 537–542

Cutler, R. E. & B M Orme Correlation of serum creatinine concentration and kanamycin half life *J amer med Ass* 1969, 209, 539–542.

Dettli, L. & P Spring Factors influencing drug elimination in man *Il Farmaco* (Sc. ed.) 1968, 23, 795–812

Dettli, L. *Multiple dose elimination kinetics and drug accumulation in patients with normal and with impaired kidney function* In *Advances in the biosciences* 5, pp 39–54 Ed. *G Raspé Pergamon Press* and Vieweg, Oxford, New York and Braunschweig 1970a

Dettli, L. Pharmakokinetik bei repetierter Arzneimittelapplikation Kumulationslehre In *Lehrbuch der klinischen Pharmakologie und Toxikologie* Ed E Garret & H Spitzy Urban and Schwarzenberg, Munchen Berlin Wien, 1970b, pp 59–64

Dettli, L. Dosierungstheorie fur die repetierte Applikation reversibel wirkender Pharmaka bei Eliminationsinsuffizienz. In *Pharmacological and clinical significance of pharmacokinetics* Symposia Medica Hoechst, F K. Schattauer, Stuttgart New York 1970c, pp 31–41

Dettli, L. Drug dosage in patients with impaired kidney function. *Postgrad med J* 1970d, 46, Suppl 32–35

Doherty, J E. W H. Perkins & M C. Wilson Studies with tritiated digoxin in renal failure. Amer med J 1964, 37, 536–544

Doherty J E, W J Flanigan, W H. Perkins & G L. Ackermann Studies with tritiated digoxin in anephric human subjects *Circulation* 1967, 35, 298–303

Doherty, J E, W J Flanigan & W H Perkins Tritiated digoxin excretion of patients *following renal transplantation Circulation 1968, 27, 865–869*

Doherty, J E., W J Flanigan, R. M Patterson & G V Dalrymple The excretion of tritiated digoxin in normal human volunteers before and after unilateral nephrectomy *Circulation 1969, 40, 555–561*

Ewy, G A., G G Kapadia, L. Yao, M Lullin & F I Marcus Digoxin metabolism in the elderly *Circulation* 1969, 39, 449–453

Fabre J., J S Pitton, C. Virieux F L. Laurencet, J P Bernhardt & J G Godel La doxycycline: absorption, distribution et excretion d'un nouvel antibiotique à large spectre chez l homme *Schweiz med Wschr* 1967, 97, 915–924

Gingell, I C. & P M Waterworth Dose of gentamycin in patients with normal renal function and renal impairment. *Brit med J* 1968, II, 19–22.

Goodwin N J & E A. Friedman *The effect of renal impairment, peritoneal dialysis,* and hemodialysis on sodium colistimethate levels *Ann intern Med.* 1968, 68, 984–994

Healy J K. Clinical assessment of glomerular filtration rate by different forms of creatinine clearance and a modified urinary phenolsulphonthalein excretion test. *Amer J Med* 1968, 44, 348–358.

Jelliffe, R. W A mathematical analysis of digitalis kinetics in patients with normal and reduced renal function *Math Biosciences* 1967, 1, 305–325

Kabins, S A. & S Cohen Cephalothin serum levels in the azotemic patient *Antimicrobial Agents and Chemotherapy* 1964, 4, 207–214

Kabins, S A &S Cohen Cephalondine therapy as related to renal function *Anti microbial Agents and Chemotherapy* 1965, 5, 922–932

Kramer, P, B Willms, J Horenkamp & F Scheler Blutspiegelkinetik und renale Clearance von ³H Peruvosid *Klin Wschr* 1969, 47, 1157–1166

Kramer, P, J Horenkamp, B Willms & F Scheler Das Kumulationsverhalten verschiedener Herzglykoside bei Anurie *D med Wschr* 1970, 95, 444–453

Kruger-Thiemer, E. Dosage schedule and pharmacokinetics in chemotherapy *J amer pharm Ass* (Sc ed) 1960, 49, 311–313

Kunin, C M & M Finland Restrictions imposed on antibiotic therapy by renal failure *AMA Arch intern Med* 1959a, 104, 204–224

Kunin, C M & M Finland Persistence of antibiotics in blood of patients with acute renal failure III Penicillin, streptomycin, erythromycin and kanamycin *J clin Invest* 1959b, 38, 1509–1519

Kunin, C. M, S B Rees, J P Merrill & M Finland Persistence of antibiotics in blood of patients with acute renal failure I Tetracycline and chlortetracycline *J clin Invest* 1959a, 38, 1487–1497

Kunin, C M, A J Glatzko & M Finland Persistence of antibiotics in blood of patients with acute renal failure. II Chloramphenicol and its metabolic products in the blood of patients with severe renal disease or hepatic cirrhosis *J clin Invest* 1959b, 38, 1498–1508

Kunin, C M & N Atul Excretion of cephalondine and cephalothin in patients with renal impairment *New Engl J Med* 1966 274, 654–656

Kunin, C M Problems of antimicrobial drug therapy in renal failure Proc 3rd int Congr Nephrol 3, 193–213 Karger, Basel New York, 1967a

Kunin, C M A guide to use antibiotics in patients with renal disease *Ann intern Med* 1967b, 67, 151–158

Kunin, C. M More on antimicrobials in renal failure *Ann intern Med* 1968 69 397–398

Lahrtz, H & P A van Zwieten The influence of kidney or liver disorders on the serum concentration and urinary excretion of ³H peruvoside, a tritium labelled cardiac glycoside. *Eur J Pharmacol* 1968 3, 147–152

Lindberg, A A, L. H Nilsson, H Bucht & L O Kallings Concentration of chloramphenicol in the urine and blood in relation to renal function *Brit med J* 1966 II, 724–728.

Naumann, P & W Auwarter Die parenterale Colistin Therapie Wirkstoffspiegel und antibakterielle Aktivitat Proc 3rd int Congr Chemother 1, 311–318 Thieme Stuttgart, 1964

Orme, B M & R E Cutler The relationship between kanamycin pharmacokinetics distribution and renal function *Clin Pharmacol Therap* 1969 10, 543–550

Pryor, J S, A M Joekes & R D Foord Cephaloridine excretion in patients with normal and impaired renal function *Postgrad med J* 1967 43 suppl 82 85

Reubi, F & C. Munger Renale und extrarenale Ausscheidung von N pyrrolidinomethyltetracyclin (Reverin) bei Patienten mit normaler und eingeschrankter Nieren funktion *Pharmacologia Clinica (Berl)* 1968 1 8–18

Reubi, F & C. Vorburger Renale und extrarenale Ausscheidung von Ampicillin bei Patienten mit normaler und eingeschrankter Nierenfunktion *Pharmacologia Clinica* (Berl) 1969, 1, 192–202

Yamasaku, F, R. Tsuchida & Y Usuda The kinetics of cephalosporins in renal impairment *Postgrad med J* 1970 46, Suppl 57–59

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Pathophysiological Factors Influencing Drug Kinetics

By

Alasdair Breckenridge

Many early pharmacokinetic studies were done in a variety of animal species and extrapolation of these results to man was common It has become evident, however, that for many drugs, kinetic data so obtained frequently bears little or no relationship to those found in man and this lead to the policy of doing drug studies in man at an early stage in the development of a new drug (BRODIE 1964) For several years, physicians have used kinetic data obtained in healthy volunteers to calculate drug doses and frequency of dosing in patients with systemic disease Often the comparison between healthy volunteers and ill patients is as inappropriate as comparison between species

When discussing the ways in which disease can influence drug kinetics, one must remember the wide individual variation in kinetic values encountered in normals, e g in rates of drug metabolism It is thus not surprising that when in disease there is again wide variation in these parameters, the normal and abnormal may overlap completely

What is required to study kinetics in ill patients are conscientious longitudinal studies of a few patients as their disease progresses, satisfactorily or not, rather than single observations in patients at widely differing stages of apparently the same disease This is difficult for two reasons Firstly there is a natural reluctance to investigate patients who may be in considerable discomfort, and procedures such as repeated venepunctures which appear trivial to the medical scientist who has a vested interest in the results, may be very unwelcome to the patient. Secondly, patients with diseases are frequently treated with more than one drug simultaneously A recent survey at Hammersmith Hospital has shown that the average number of drugs given to inpatients simultaneously was four and one in twenty patients was taking ten or more drugs at the same time The record number was thirty drugs given to one patient in a 24 hour period The effects of the simultaneous administration of more than one drug on kinetic processes is well known

Changes in drug absorption

MARTIN (1964) has shown wide individual variations in the absorption of salicylates, which may be related to changes in the physical nature of the intestinal tract, or to changes in gut motility

Recently FABRE *et al* (1968) studied the pharmacokinetics of the tetracycline analogue doxycycline after oral administration He found a plasma half-life of 22 hours, which compared with other tetracyclines whose plasma half-life varied between 8 and 10 hours The reason for this variation was that doxycycline, unlike the other tetracyclines was extremely slowly absorbed and thus the elimination process was influenced by the simultaneous absorption of the drug

In disease the number of studies of the effect of disease on drug absorption is small DAVIES & PIROLA (1968) have shown that the peak plasma levels of phenoxymethylpenicillin in patients with gluten sensitive enteropathy after a dose of 500 mg of drug was reduced up to ten fold as compared with normal subjects In steatorrhoea from other causes, e g post gastrectomy or pancreatic disease, there was no apparent change in penicillin absorption While plasma curves do not give a quantitative measurement of absorption, the resultant plasma concentration of antibiotics after oral administration are of the utmost clinical importance It would thus appear rational to administer antibiotics such as penicillin to patients with an active enteropathy, by the parenteral route SCHEDL & CLIFTON (1963) showed that in the normal individual there is a gradient within the small intestine for cortisol absorption from jejunum to ileum In a patient with active sprue, this gradient was abolished, but treatment with a gluten-free diet for a period of 3 months restored normality

It is of interest to speculate that with the increasing use of agents such as neomycin used to lower plasma lipids, preventing their absorption by interrupting micellar aggregates (THOMPSON *et al* 1970), iatrogenic malabsorption of drugs may become of increasing importance, an aspect which currently concerns us

Changes in drug distribution

The elimination of many drugs can be described as a first order process

$$-\frac{dC}{dt} = KC, \text{ where } C = \text{concentration of drug in plasma}$$

Now K, the slope of the plasma elimination curve, can also be expressed in terms of the clearance of the drug from the plasma and its apparent volume of distribution

$$\text{Thus } K = \frac{\text{Clearance of drug from plasma}}{\text{Volume of distribution}}$$

$$\text{Thus } -\frac{dC}{dt} = \frac{\text{Clearance}}{\text{Volume of distribution}} \times C$$

Since only free drug (i e unbound) can be eliminated, a factor, α = fraction of drug unbound must also be considered

$$\text{Thus } -\frac{dC}{dt} = \frac{\text{Clearance} \times C \times \alpha}{\text{Volume of distribution}}$$

It has been shown mathematically that

$$\alpha = \frac{P + K_D + D_F}{K_D + D_F}$$ where K_D = dissociation constant for drug protein, P = concentration of protein, D_F = concentration of unbound drug

Thus as P, the concentration of protein decreases, it might be expected that α, will increase If α increases, then $\frac{dC}{dt}$, the rate of elimination of the drug will also increase But if α increases it might be predicted that the volume of distribution will increase, so the change in the rate of elimination of the drug might not change greatly

There are few studies of how diseases may effect drug kinetics by changes in protein concentration WYERS & VAN MUNSTER (1961) showed that patients with nephrotic syndrome with low plasma albumen eliminated the dye Evans Blue more quickly than normals REYNOLDS & CLUFF (1960) examined the effects of a variety of diseases on salicylate binding by plasma albumin They showed that binding was decreased at concentration of salicylate varying from 50–200 μg/ml and that these changes correlated well with changes in plasma albumin concentration

Changes in drug distribution have also been postulated as the reason for the variation in digoxin sensitivity and requirements in patients with thyroid disease (DOHERTY & PERKINS 1966) Hyperthyroid patients showed consistently lower plasma digoxin levels and hypothyroid patients, higher levels than euthyroid subjects after similar doses of the drug. The dominant digoxin half-life was not different in the 3 groups but when the second exponential of the serum curve was studied, hyperthyroid subjects showed a more rapid, and hypothyroid subjects a less rapid decay than euthyroid subjects

Changes in drug metabolism

Many factors are alleged to have an influence on the metabolism of drugs by the liver in man

1 *Genetic influences* Data such as that obtained by ALEXANDERSSON *et al* (1969) and VESELL & PAGE (1968) on identical and fraternal twins show the importance of genetic influences in drug metabolism

2 *Age* In the neonatal period, drug metabolising activity in the liver is low (FOUTS & ADAMSON 1959) but whether this is due to a deficiency of the enzyme or an inhibition of enzyme by gonadal hormones is disputed There is no evidence that the rate of drug metabolism changes with increasing age, but no longitudinal studies have been done in man

3 *Sex* As there is a wide range of rates of drug metabolism in both sexes, sex differences would be difficult to find The observation that norethynodrel and progesterone, both used as contraceptive steroids, will inhibit drug metabolism *in vitro* (JUCHAU & FOUTS 1966) may be important in individual women

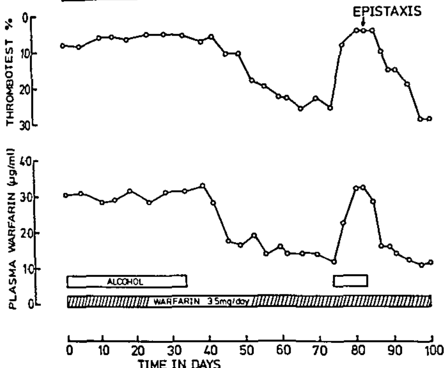
4 *Pregnancy* CRAWFORD & RUDOFSKY (1966) showed that in pregnant females the rate of metabolism of promazine and pethidine was slower than in non-pregnant females NEALE & PARKE (1969) have shown that rates of hydroxylation of diphenyl was decreased in pregnant rats and this correlated with a change in cytochrome P-450 content in the liver

5 *Environment* Workers exposed to an environment where they come in contact with an inducer of drug metabolising enzymes have been shown to have a shorter plasma antipyrine half-life than controls (KOLMODIN *et al* 1969)

6 *Smoking* Although cigarette smoking has been shown to induce the formation of benzpyrene hydroxylase in the placenta (WELCH *et al* 1969) and to induce the metabolism of nicotine (BECKETT & TRIGGS 1967) there is no evidence that cigarette smoking is associated with gross differences in rates of drug metabolism (VESELL & PAGE 1968)

7 *Ethanol* It has recently been shown that ethanol is metabolised in the liver by two mechanisms – alcohol dehydrogenase which is a cyto plasmic enzyme, and by the microsomal oxidizing system Furthermore chronic ethanol administration has been shown to increase the activity of pentobarbitone (mebumalum NFN) hydroxylase, benzpyrene hydroxylase and anilene hydroxylase in rats (RUBIN & LIEBER 1968) In man the same authors showed that long term ethanol administration increased the activity of pentobarbitone hydroxylase in liver biopsy samples of 3 human volunteers given ethanol for 12 days

We have studied the effects of ethanol administration to patients on long term anticoagulant therapy The most striking change was shown in



one patient whose plasma warfarin level fell from 3.0 to 1.2 µg/ml on withdrawal of ethanol with a corresponding change in anticoagulant control, and on reintroduction of ethanol into his diet, plasma warfarin level rose again to 3.2 µg/ml, thrombotest (Owren) fell to below 5 % and the patient suffered an epistaxis. On withdrawal of alcohol the plasma warfarin

Table I

Table 1 Liver disease and drug metabolism

Difference found	*No difference found*
Chloramphenicol – KUNIN *et al* (1959)	Pentobarbitone – SESSIONS *et al* (1959)
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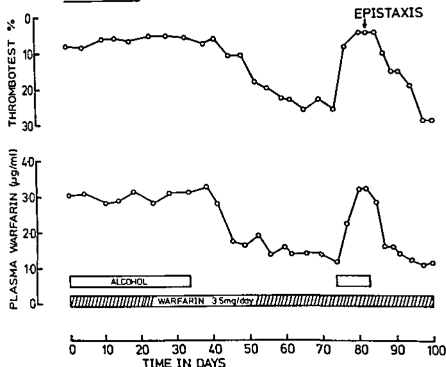
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Changes in drug excretion

Drugs which are largely cleared by renal excretion show a prolongation of plasma half-life in patients with renal impairment For digoxin which is excreted largely by glomerular filtration (DOHERTY *et al* 1964) a good correlation has been shown between creatinine clearance as a measure of glomerular filtration rate and the urine excretion of tritiated digoxin With the few exceptions such as penicillin and the metabolic products of chloramphenicol, most antibiotics in current use are cleared by the kidney by glomerular filtration, and thus patients with impaired renal function will show a prolonged plasma drug half life and thus the dose required need be given less frequently The plasma half life for penicillin has been shown to be prolonged from 30 minutes in normals to 7–10 hours in anuric patients (KUNIN & FINLAND 1959) In anuria, liver inactivation of penicillin becomes important, and the same authors studied two anuric patients with liver disease in whom the plasma half life was prolonged to 16 4 and 30 5 hours respectively

REFERENCES

Alexanderson, B , D A Price & F Sjoqvist Steady state plasma levels of nortriptyline in twins Influence of genetic factors and drug therapy *Brit Med J* 1969, 4, 764

Beckett, A H & E J Triggs Enzyme induction in man caused by smoking *Nature* 1967, 216 587

Breckenridge A, M Orme, D Davies & S Thorgeirsson Induction of drug metabolising enzymes in man and rat by dichloralphenazone *Clin Sci* 1969, 37, 565

Brodie, B B In *Absorption and distribution of drugs*, p 199 Ed T Binns, Livingstone Edinburgh. 1964

Brodie, B B, J J Burns & M Weiner Metabolism of drugs in subjects with Laennec's cirrhosis Med Exp 1959, 1 290

Crawford, J S & S Rudofsky Some alterations in the pattern of drug metabolism associated with pregnancy, oral contraceptives and the newly born *Brit J Anaesth* 1966, 38 446

Davies, A E & R C Pirola Absorption of phenoxymethylpenicillin in patients with steatorrhoea *Aust Ann Med* 1968, 17, 63

Doherty, J E & W H Perkins Digoxin metabolism in hypo and hyperthyroidism *Ann Int Med* 1966, 64, 489

Doherty, J E W H Perkins & M C Wilson Studies with tritiated digoxin in renal failure *Amer J Med Sci* 1964, 37, 536.

Fabre, J, J P Kunz, C Virieux, J L Laurencet & J S Pitton *Le comportement de la doxycycline chez l'homme Chemotherapie* 1968, 13, 23

Fouts, J R & R H Adamson Drug metabolism in the newborn rabbit *Science* 1959, 129, 897

Juchau, M R & J R Fouts Effect of norethynodrel and progesterone on hepatic microsomal drug metabolising enzyme systems *Biochem Pharmacol* 1966, 15, 891

level again fell and thrombotest percentage rose (fig 1) This patient had mildly impaired liver function as shown by B S P retention of 9 5 % both when on and off alcohol Whether the same phenomenon can be shown in subjects with normal liver function is under study

8 *Liver disease* There is much controversy whether disease of the liver can alter rates of drug metabolism in man Table 1 summarises data obtained from several authors some of whom have and others have not found changes in rates of drug oxidation The problem is that there is a range of rates of drug metabolism in man and a large overlap undoubtedly exists between normal patients and patients with liver disease An attempt to differentiate the effects of prior drug administration in patients with liver disease from the effects of the liver disease itself was made by LEVI *et al* (1968) studying the plasma half life of phenylbutazone given orally as a single dose When they examined the drug history of patients with liver disease they found that a proportion were taking agents known to induce drug metabolism in man The mean plasma half life of phenylbutazone in normal subjects was 78 1 ± 5 hours and in normal subjects taking inducing agents the mean plasma half life fell to 57 2 ± 8 hours In patients with liver disease who were not taking inducing agents the mean plasma phenylbutazone half life was 100 ± 10 hours and in those on inducing agents 54 3 ± 5 hours When one studies the drugs and the dose considered as significant pretreatment with inducing agents it would appear from our steady state studies on patients on long term warfarin therapy (BRECKENRIDGE *et al* 1969) that some of the drug doses quoted are unlikely to induce drug metabolising enzymes significantly

Induction of drug metabolising enzymes

Many agents are known to increase the rate of drug metabolism We have studied the effects administering several widely used hypnotic agents to patients on long term warfarin therapy One of the most interesting hypnotics we have studied is dichloralphenazone a molecular complex of chloral hydrate and phenazone (antipyrine) Dichloralphenazone we found to be a powerful inducer of warfarin metabolism and this effects was not due to chloral hydrate the hypnotic ingredient but to antipyrine added to chloral hydrate to render it more palatable Measurement of the plasma antipyrine half life in patients given dichloralphenazone gave a range from 7 to 25 hours Those patients with a long plasma antipyrine half life showed a greater fall in plasma warfarin level when given dichloralphenazone and there was a significant correlation between the degree of induction with dichloralphenazone and the length of the plasma antipyrine half life

From the Chemical Research Laboratories, Pharmaceutical Division, CIBA Limited, Basle, Switzerland

Blood Levels of a Schistosomicide in Relation to Liver Function and Side Effects

By

Johann W. Faigle

Ambilhar® is an antiparasitic agent employed chiefly for the treatment of bilharziasis or schistosomiasis, a widespread tropical disease The preparation is effective by the oral route and only needs to be administered for 5 to 10 days This advantage of a short and simple dosage schedule also makes ambilhar suitable for use in mass-treatment programmes (SCHMIDT & WILHELM 1966, FONTANILLES 1969)

The drug is now being employed mainly in two regions of the world in Africa, where bilharziasis due to *S haematobium*, as well as to *S mansoni*, is endemic, and in Latin America, where only the latter form of bilharziasis occurs Experience acquired in several thousand patients has shown that in Africa ambilhar® is generally well tolerated By contrast, in Latin America, central nervous side-effects, which necessitate withdrawal of the medication occur relatively frequently (FONTANILLES 1969)

The purpose of the present paper is to describe briefly how, with the aid of a detailed metabolic study, an explanation for this surprising geographical difference was found The study in question involved some 50 patients with bilharziasis, and comprised three separate series of investigations carried out in Portugal, Rhodesia, and Brazil Owing to the special nature of the analytical techniques employed, it was necessary for us to travel to these countries ourselves in order to tackle the work on the spot in co-operation with local clinics (FAIGLE & KEBERLE 1969, FAIGLE *et al* 1970a & b)

The pharmacokinetics of ambilhar®

The first series, consisting of only a few test subjects, yielded at the start a general picture of the drug's pharmacokinetic properties, which can be summarized as follows

The active substance of ambilhar® belongs chemically to the category

Kolmodin, B, D L Azarnoff & F Sjoqvist Effect of environmental factors on drug metabolism *Clin Pharmacol Therap* 1969, **10**, 638

Kunin, C M & M Finland Persistence of antibiotics in blood of patients with acute renal failure *J Clin Invest* 1959, **38**, 1509

Kunin, C M, A J Glazko & M Finland Persistence of antibiotics in blood of patients with acute renal failure ii Chloramphenicol and its metabolic products in the blood of patients with severe renal disease or hepatic cirrhosis *J Clin Invest* 1959, **38**, 1498

Kutt, H, W Winters & R Scherman Diphenylhydantoin and phenobarbital toxicity *Arch Neurol* 1964, **11**, 649

Laidlaw, J, A E Read & S Sherlock Morphine tolerance in hepatic cirrhosis *Gastroenterology* 1961, **40**, 389

Levi, A J, S Sherlock & D Walker Phenylbutazone and isoniazid metabolism in patients with liver disease in relation to previous drug therapy *Lancet* 1968 **1** 1275

Martin, B K In *Absorption and distribution of drugs*, p 165 Ed T Binns, Livingstone Edinburgh, 1964

Neale, M G & D V Parke The effect of pregnancy on the hydroxylation and reduction of drugs and cytochrome P-450 content of rat liver microsomes *Biochem J* 1969, **113**, 12P

Nelson, E Rate of metabolism of tolbutamide in test subjects with liver disease or with impaired renal function *Amer J Med Sci* 1964, **248**, 657

Reynolds, R C & L E Cluff Interaction of serum and sodium salicylate *Bull John Hopkins Hosp* 1960, **107**, 278

Rubin, E & C S Lieber Hepatic microsomal enzymes in man and rat, induction and inhibition by ethfanol *Science* 1968, **162**, 690

Schedl, H P & J A Clifton Cortisol absorption in man *Gastroenterology* 1963, **44**, 134

Sessions, J T, H P Minkel, J C Bullard & F J Ingelfinger The effect of barbiturates in patients with liver disease *J Clin Invest* 1959, **33**, 1116

Thompson, G R, M McMahon & P Claes Precipitation by neomycin compounds of fatty acids and cholesterol from mixed micellar solutions *Europ J Clin Invest* 1970, **1**, 40

Vesell, E S & J G Page Genetic control of dicoumarol levels in man *J Clin Invest* 1968, **47**, 2657

Welch, R M, Y E Harrison, B W Gommi, P J Poppers, M Finster & A H Conney Stimulatory effect of cigarette smoking on the hydroxylation of 3 4 Benzpyrene by enzymes in the human placenta *Clin Pharmacol Therap* 1969, **10**, 100

Wyers, J H & P J J van Munster The disappearance of Evans Blue dye from the blood in normal and nephrotic subjects *J Lab Clin Med* 1961, **58** 375

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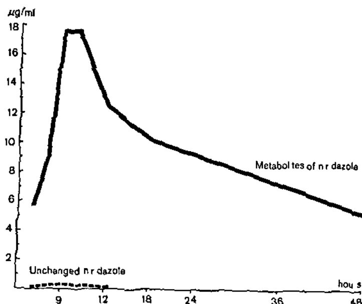


Fig 1 Blood levels of niridazole* and its metabolites in man following a single dose (25 mg/kg)

of the nitrothiazoles (1 (5-nitro-2-thiazolyl)-2-imidazolidinone Proposed international non-proprietary name niridazole) Its fate is characterized by slow absorption from the gastro-intestinal tract and rapid degradation in the liver Its slow rate of absorption is due to the fact that the substance is only sparingly soluble in aqueous media, i e an orally administered dose acts like a depot, from which the substance is released over a period of several hours Because the drug is quickly broken down in the liver the concentration of active substance in the blood remains relatively low Some of the metabolites are strongly bound to the serum albumin, with the result that they attain considerably higher and more prolonged blood concentrations These metabolites display no therapeutic activity

The typical pattern of the concentration-time curves in man plotted after administration of a single oral dose of 25 mg/kg is shown in fig 1 Ambilhar® was measured in *whole* blood ^{14}C-labelled material was used Total ^{14}C was measured in each sample and the unchanged drug was measured separately by inverse isotope dilution analysis

The dosage schedule for ambilhar®

On the basis of these preliminary results indicating how long the parent drug remained in the blood, a dosage schedule providing for two daily doses given at 12-hour intervals was proposed In the light of observations

* Proposed international non-proprietary name for the active substance of ambilhar®

made during therapeutic trials, the duration of treatment was fixed at 5 to 10 days, and the daily dosage at 25 mg/kg

A second, more extensive series of metabolic studies was undertaken on indigenous African patients in Rhodesia In this country, only mild forms of bilharziasis are usually met with, i e forms in which the disease has not yet produced any irreversible organic lesions (BLAIR & CLARKE 1969)

The uniformity of the pharmacokinetic parameters was first checked in 4 groups composed of 3 patients each From a previous course of treatment, it was already known how these patients had responded to ambilhar® Each person was given another single dose of 25 mg/kg The blood concentrations of parent compound and its metabolites are shown in fig 2 in the form of average curves for each of the 4 groups None of the criteria applicable to these groups (occurrence of mild side-effects, failure to achieve a complete cure, age of the patient) could be linked to any special pharmacokinetic pattern The differences observed were very minor as were the interindividual fluctuations (not plotted here) It should be added that these results largely confirmed the validity of the dosage schedule originally proposed

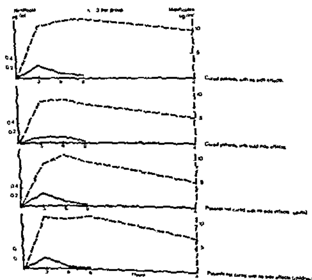


Fig 2 Concentration of niridazole and its metabolites in the blood of patients following a single oral dose of 25 mg/kg (*S mansoni* patients who had undergone a full course of treatment with niridazole *three months* previously)

—— Niridazole

– – – Metabolites

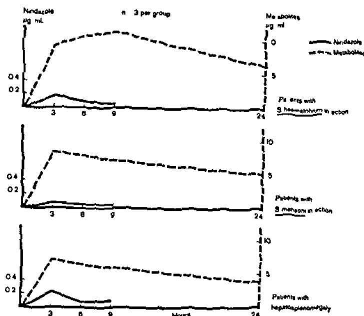


Fig 3 Concentration of niridazole and its metabolites in the blood of patients following a single oral dose of 25 mg/kg (patients who had not previously been treated with niridazole)

Three further groups, each consisting of 3 Rhodesian patients, were investigated in order to determine the possible influence of the disease on the drug's kinetics Included among these patients were persons suffering from uncomplicated *S haematobium* and *S mansoni* bilharziasis, as well as cases of advanced bilharziasis associated with hepatosplenomegaly

In the patients in the last group (fig 3) the rate of metabolism of niridazole seemed to be slower than in subjects with uncomplicated bilharziasis *Thus the concentrations of parent drug, as compared with those of the* respective metabolites, were higher than in the other groups of patients

As this difference could not be confirmed statistically, however, a third *series of studies was carried out, this time in South America*

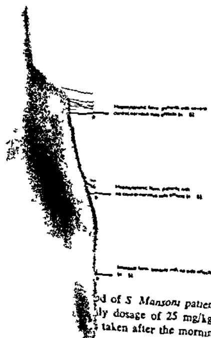
Ambilhar® in patients with advanced bilharziasis

The North-Eastern region of Brazil was chosen as a suitable area in which to undertake these investigations, because here the percentage incidence of severe, so called hepatosplenic forms of bilharziasis is far higher than in Africa In the meantime, it had also been discouvered that in such cases ambilhar® produced disturbing side-effects (COUTINHO & TRINDADE BARRETTO 1969, FONTANILLES 1969) The question consequently arose of possibly altering the dosage schedule in hepatosplenic patients The persons

studied comprised a group of 12 patients suffering from the hepatosplenic form of *S mansoni* bilharziasis, as well as a control group of 5 patients with (uncomplicated) intestinal *S mansoni* bilharziasis Here, the analyses were performed while the patients were receiving a therapeutic course of ambilhar® medication lasting several days, the samples required for analysis being taken after the morning dose The treatment had to be withdrawn in 6 of the hepatosplenic cases, owing to side-effects such as convulsions hallucinations and confusion, in none of the patients in the control group, however, was withdrawal of the medication necessary

In fig 4 the concentrations of active substance in the blood are recorded in the form of individual and average curves The patients in the first group have been divided into two subgroups, depending on whether or not side effects occurred The concentrations of the metabolites have been omitted, because these showed no group-specific peculiarities

From the curves presented here it can be seen that the patients with hepatosplenic forms of the disease – including especially those who develop... e effects – had considerably higher concentrations of active sub- ...r blood than the patients in the control group The difference ... statistical significance ($P < 0.01$) From this finding it can ... t, in advanced forms of the disease, the bioinactivation



...d of *S Mansoni* patients on the 3rd or ...ly dosage of 25 mg/kg administered in ... taken after the morning dose)

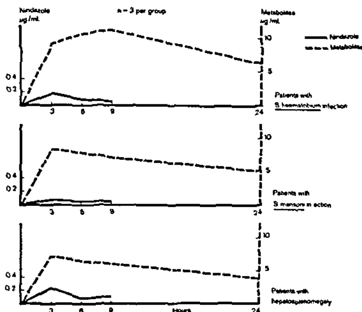


Fig 3 Concentration of niridazole and its metabolites in the blood of patients following a single oral dose of 25 mg/kg (patients who had not previously been treated with niridazole)

Three further groups, each consisting of 3 Rhodesian patients, were investigated in order to determine the possible influence of the disease on the drug's kinetics Included among these patients were persons suffering from uncomplicated *S haematobium* and *S mansoni* bilharziasis, as well as cases of advanced bilharziasis associated with hepatosplenomegaly

In the patients in the last group (fig 3) the rate of metabolism of niridazole seemed to be slower than in subjects with uncomplicated bilharziasis Thus the concentrations of parent drug, as compared with those of the respective metabolites, were higher than in the other groups of patients

As this difference could not be confirmed statistically, however, a third series of studies was carried out, this time in South America

Ambilhar® in patients with advanced bilharziasis

The North-Eastern region of Brazil was chosen as a suitable area in which to undertake these investigations, because here the percentage incidence of severe, so called hepatosplenic forms of bilharziasis is far higher than in Africa In the meantime, it had also been discovered that in such cases ambilhar® produced disturbing side-effects (COUTINHO & TRINDADE BARRETTO 1969, FONTANILLES 1969) The question consequently arose of possibly altering the dosage schedule in hepatosplenic patients The persons

studied comprised a group of 12 patients suffering from the hepatosplenic form of *S mansoni* bilharziasis, as well as a control group of 5 patients with (uncomplicated) intestinal *S mansoni* bilharziasis Here, the analyses were performed while the patients were receiving a therapeutic course of ambilhar® medication lasting several days, the samples required for analysis being taken after the morning dose The treatment had to be withdrawn in 6 of the hepatosplenic cases, owing to side effects such as convulsions, hallucinations and confusion, in none of the patients in the control group, however, was withdrawal of the medication necessary

In fig 4 the concentrations of active substance in the blood are recorded in the form of individual and average curves The patients in the first group have been divided into two subgroups, depending on whether or not side-effects occurred The concentrations of the metabolites have been omitted, because these showed no group-specific peculiarities

From the curves presented here it can be seen that the patients with hepatosplenic forms of the disease – including especially those who developed side effects – had considerably higher concentrations of active substance in their blood than the patients in the control group. The difference is also of high statistical significance ($P < 0\,01$) From this finding it can be concluded that, in advanced forms of the disease, the bioinactivation

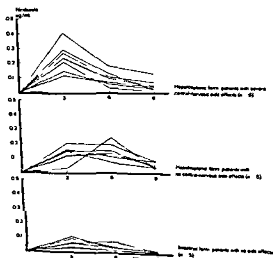


Fig 4 Concentration of niridazole in the blood of *S Mansoni* patients on the 3rd or 4th day of treatment lasting several days (daily dosage of 25 mg/kg administered in two fractional doses, blood specimens taken after the morning dose)
——— Individual curves
/ / Average curves

of ambilhar® is impaired At the same time, however, it must be noted that the metabolism of the drug in such patients shows large individual differences It is therefore questionable whether a general reduction in the dosage of ambilhar® in hepatosplenic cases would result in a consistently better degree of tolerability coupled with the maintenance of a satisfactory therapeutic effect

The decrease in the rate at which ambilhar® is broken down in such patients is probably attributable, not so much to an enzymatic defect in the liver, but rather to the existence of so-called portal-systemic shunts Shunts of this kind often develop as a consequence of hepatosplenic bilharziasis, in some of our cases in which side-effects occurred, it was also possible to demonstrate the presence of these shunts by X-ray examination (COUTINHO & TRINDADE BARRETTO 1969) When ambilhar® is given to these patients, a portion of the substance absorbed by-passes the liver and flows directly into the peripheral circulation, it can thus no longer be rapidly metabolized as in normal cases The resultant higher concentration of active drug in the peripheral blood offers a ready explanation for the higher incidence of side-effects in such patients

Summary

The state of the disease to be treated influences the pharmacokinetic of ambilhar® (niridazole), a schistosomicide In mild cases of bilharziasis, which are very common, the blood levels of the parent drug show only slight interindividual variations In such patients, the therapeutic effectiveness and tolerability of the preparation are usually uniformly good

In patients suffering from advanced forms of bilharziasis considerably higher blood levels and greater interindividual fluctuations are encountered, resulting in a higher incidence of side-effects Since portal-systemic shunts are liable to develop in such patients it is suggested that a portion of the drug upon absorption by-passes the liver, which results in a slower metabolism

REFERENCES

Blair, D M & V de V Clarke Field studies in Rhodesia *Ann N Y Acad Sci* 1969 160, 915–929

Coutinho, A D & F Trindade Barretto Treatment of hepatosplenic schistosomiasis mansoni with niridazole. *Ann N Y Acad Sci* 1969, 160, 612–628

Faigle, J W & H Keberle Metabolism of niridazole in various species including man *Ann N Y Acad Sci* 1969, 160, 544–557

Faigle J W., D M Blair, V de V Clarke, L. A. G Davidson, D C. Dukes & H Keberle The metabolism of ambilhar® in man I Investigations on patients suffering from clinically mild forms of *S haematobium* and *S mansoni* bilharziasis *Ann Trop Med Parasitol* 1970a, 64, 373–382.

Faigle, J W., A D Coutinho, H Keberle & F Trindade Barretto The metabolism of ambilhar® in man. II Investigations on patients suffering from clinically advanced forms of *S mansoni* bilharziasis *Ann Trop Med Parasitol* 1970b, 64, 383–393

Fontanilles, F Risks versus benefits in antischistosomal therapy *Ann N Y Acad Sci* 1969, 160, 811–820

Schmidt, P & M Wilhelm Eine neue Wirkstoffgruppe gegen Bilharziose *Angew Chem* 1966 78, 850–855

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Developmental Aspects of Pharmacokinetics

By

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Abstract The absorption, apparent volumes of distribution, serum concentrations, serum half lives, and urinary excretion of several antibiotics were studied in premature infants by means of a micro diffusion assay technique Serum half lives of all drugs investigated (except colistin) were markedly prolonged during the first days and weeks of life and approached adult values by three weeks of age These changes were paralleled by an increase in urinary excretion rate which was independent of birth weight and gestational age Colistin had a mean serum half life in premature infants of 2 4 hours comparable to that noted in adults and thus is a notable exception Studies of hepatic microsomal suspensions derived from human foetuses have revealed the presence of the component members of both the NADPH and the NADH linked electron transport systems Exogenous substrates such as aminopyrine and benzpyrene were not significantly metabolized by human foetal liver suspensions but the endogenous compounds testosterone and laurate, were hydroxylated at substantial rates The binding of these substrates to cytochrome P 450 was investigated by studying the spectral changes that occur when they were added to microsomal suspensions Laurate and testosterone exhibited type I changes whereas aminopyrine gave rise to a type II spectral change correlating with the low or absent demethylating activity in foetal liver These findings in the human foetus stand in marked contrast to results obtained in animals and emphasize again the marked species differences *which exist in drug metabolism*

The developing foetus and newborn infant are thought to be more susceptible to the action of drugs and exogenous agents than the adult organism This belief has arisen from clinical experience in which drug administration to either the pregnant woman or the newborn infant has been associated with unexpected adverse and at times fatal reactions (WEISS *et al* 1960, NYHAN & LAMPERT 1965) Several recent review articles (YAFFE 1966 MIRKIN 1970) have surveyed the current state of knowledge in this area which has been called Developmental or Perinatal Pharmacology The lack of data in man prompted us to initiate detailed investigations in the human foetus and in the immature infant The following studies have been carried

out in our laboratories over the past several years and represent a brief look at the pharmacokinetics of the elimination process in the premature infant and foetal liver They are described separately for the sake of clarity in presentation, but both processes (metabolism and excretion) operate together *in vivo* and must be considered as such in prescribing drugs to the young infant

Pharmacokinetics of antibiotic substances in premature infants

The absorption, apparent volumes of distribution, serum concentration and urinary excretion of several non metabolizable antibiotics were studied in premature infants by means of a micro-diffusion technique (AXLINE *et al* 1967) The infants received the drugs on therapeutic indications, fig. 1 shows the mean serum ampicillin concentrations determined in 35 premature infants of different weights and gestational ages after a single intramuscular injection of 10 mg/kg Peak serum concentrations of 13 3 to 19 4 μg/ml were reached 1 hour after injection in all infants Serum concentrations decreased slowly but exceeded 1 μg/ml for the twelve hours after injection in all infants less than 14 days of age The infants were grouped according to postnatal age and the serum half life for each group calculated from the rate constants for the decrease in serum concentrations in individual

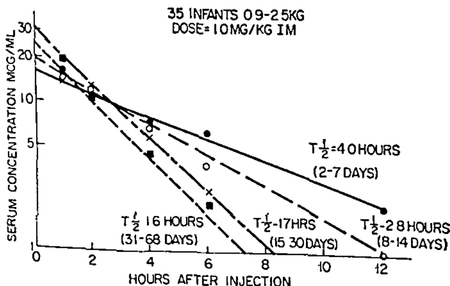


Fig. 1 Mean serum ampicillin concentrations in four groups according to age and serum half lives following single intramuscular injections of 10 mg/kg (from AXLINE *et al* 1971)

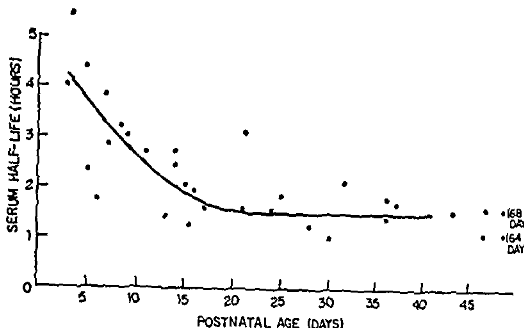


Fig 2. Correlation of individual serum half lives of ampicillin with postnatal age (from AXLINE *et al* 1967)

infants The group serum half-lives are also indicated in fig 1 and appear to decline with increasing postnatal age

The correlation of all of the individual serum half life determinations with postnatal age is shown in fig 2 The serum half life declined rapidly during the first 2 weeks of postnatal life with a less gradual change from 2 to 4 weeks and no further change beyond 1 month of age In order to determine whether the change in serum half-life were truly dependent upon postnatal age and independent of prenatal age, several infants were studied serially Despite a wide range in weights in the group (1177 to 1767 grams) the serum half-life always declined with increasing postnatal age and the rate of change was similar in all infants Since a direct correlation exists between gestational age and weight of a premature infant, these data suggest that the maturation in serum half-life values is actually a postnatal phenomenon

The apparent volumes of distribution of ampicillin, calculated by dividing the administered dose by the extrapolated serum concentration at zero time, were similar in all age groups Urinary excretion, however, increased with increasing postnatal age, so that premature infants older than 2 weeks excreted 32 % of the dose in 12 hours (adults excrete 40–60 % during the same time period) The urinary excretion of ampicillin was low when serum half lives were prolonged With increasing postnatal age, the excretion increased and serum half-lives declined These data suggest that maturation of renal mechanisms alters elimination rates and thus the serum half-lives of ampicillin Available data show that both glomerular filtration

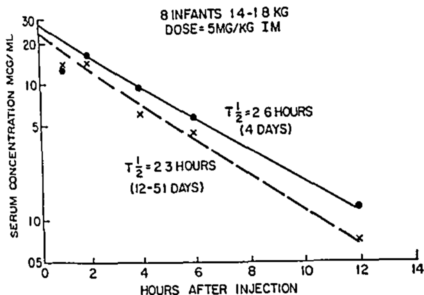


Fig 3 Mean serum concentrations of colistin according to age groups following single intramuscular injections of 5 mg/kg (from AXLINE *et al* 1967)

rate and renal tubular excretory capacity are low in premature and newborn infants and that both functions mature rapidly to adult values over several postnatal months (BARNETT *et al* 1948, WEST *et al* 1948) Our data do not define which function is more important for renal excretion of ampicillin in the premature infant, since only overall elimination into the urine was measured

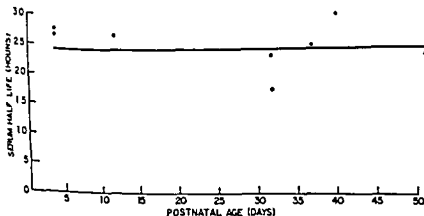


Fig. 4 Correlation of individual serum halv lives of colistin with postnatal age (from AXLINE *et al* 1967)

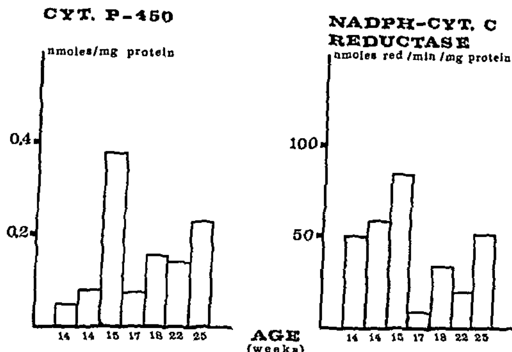


Fig 5 Content of cytochrome P 450 and activity of NADPH cytochrome c reductase *in human foetal liver* microsomes according to gestational age (in weeks)

Similar findings were noted with other penicillins (methicillin and oxacillin) and with neomycin, kanamycin, and streptomycin *A distinctly different* finding was noted with colistin following administration of a single intramuscular dose of 5 mg/kg (fig 3) Mean peak concentrations of 14 8 and 16 4 μg/ml were reached 2 hours after injection in the 2 age groups studied The mean serum half-life was 2 6 hours for two infants 4 days of age and 2 3 hours for six infants 12 to 51 days of age

The correlation of the individual serum half-life determinations for colistin with postnatal age is shown in fig 4 Although the number of patients was small, the results contrast sharply with those found with ampicillin (fig 2) and other antibiotics in that the serum half-life did not decline with increasing postnatal age Furthermore the serum half-lives do not differ from published data from which calculated half-lives were 2 0 hours *in* children (ASSAY & KOCH 1962) and 3 0 hours in normal men (WRIGHT & WELCH 1960) Glomerular filtration is the principal excretory route for colistin in adults and if similar mechanisms for elimination are operative in the premature infant, the serum half-life should have been prolonged (as it is for other antibiotics excreted primarily by filtration) These unexpected observations with colistin suggest that the drug is handled differently by the neonate and should serve as a warning not to generalize about drug excretion in the young infant

Oxidative drug metabolism in human foetal liver microsomes

Apart from antibiotics which are excreted unchanged by the kidney, the termination of action of most drug substances is accomplished by metabolic conversion in the liver cell to inactive polar metabolites. Recognition of the processes of drug metabolism as the usual rate-limiting step in the time course of drug action in the intact organism has been followed by an examination of these mechanisms in the developing organis. An investigation of the activity of glucuronyl transferase in the microsomes of guinea pig livers revealed absent or very low activity in the foetus and newborn animal (BROWN & ZUELZER 1958). Even though phenolphthalein (and not bilirubin) was used as the glucuronide acceptor and the studies were conducted in guinea pigs the results have been used to explain the elevation of unconjugated bilirubin seen in the human newborn and premature infant. Oxidative drug metabolic activity has been found to be absent in newborn rabbits (FOUTS & ADAMSON 1959) and guinea pigs and mice (JONDORF *et al.* 1958). In the latter experiments the metabolism of hexobarbital (enhexymalum NFN) was also found to be absent *in vivo* and prolonged hypnotic effects were noted in the newborn mice.

The paucity of studies of oxidative drug metabolism in the immature human liver (POMP *et al.* 1969) and the recent recognition of marked species differences in drug metabolism prompted our investigations in the human foetal liver. Another factor which provided impetus to our work was the well known and repeated observation by several endocrinologists that many steroid hormones were hydroxylated by the human foetus. In addition to determination of overall metabolism of various compounds capable of undergoing hydroxylation or oxidative demethylation we also looked for the possible presence of known components of the NADPH linked liver microsomal electron transport system, which recently have been shown to participate in the oxidation of drug substrates (COOPER *et al.* 1965, ERNSTER & ORRENIUS 1965).

Liver specimens were obtained from 13 foetuses, delivered via hysterotomy during the interruption of pregnancy for social medical reasons. Surgery was performed under halothane (or penthrane methoxiflurananum NFN), nitrous oxide and oxygen anaesthesia with morphine (or oxycodone oxyconum NFN) and scopolamine as premedication. The crown-rump lengths varied from 8.5 to 22.0 cm corresponding to gestational ages of 14 to 25 weeks. All of the specimens were placed on ice immediately at surgery. Six were studied within 5–6 hours after hysterotomy, the remainder were stored at −90 C for several weeks to months before study. No difference in the results was discernible between these two groups of livers (YAFFE *et al.* 1970).

We were unable to detect measurable hydroxylation of 3,4 benzpyrene

in any of the livers On the other hand, in five of the livers the rate of oxidative demethylation of aminopyrine varied between 2 5 and 5 9 nmoles formaldehyde formed per milligram of microsomal protein per minute There was no activity at all, however, in the five other livers examined for aminopyrine demethylation This apparent demethylating activity should be interpreted with caution because of the extremely high absorbance of the blanks obtained with microsomal preparations derived from the foetal livers Moreover, these large and highly variable blank readings made it often impossible to establish linearity between enzymic activity and the amount of micosomal protein added These results, therefore, prohibit any definitive conclusion that aminopyrine is significantly demethylated by human foetal liver microsomes and is in accord with Pomp's results and the animal investigations cited above

Since microsomal drug oxidation is medited via the catalytic component, cytochrome P-450, it was critical to assay for the presence or absence of this enzyme in the foetal microsomal preparations In previous investigations in laboratory animals, DALLNER *et al* (1966) had found only negligible amounts and activities of cytochrome P-450 and NADPH cytochrome *c* reductase before birth Therefore, it was most surprising to find highly significant amounts of cytochrome P-450 in the liver specimens in which this parameter was investigated (fig 5) The levels of the cytochrome were of the same order of magnitude as those recently found in biopsy samples of adult human liver by ALVARES *et al* (1969) and ACKERMANN (1970) It is of interest that these authors also did not find any difference in content of the cytochrome between fresh and frozen liver specimens

The flavoprotein component of the microsomal hydroxylating system, NADPH-cytochrome *c* reductase was also assayed in the same foetal liver specimens and significant activity was present (fig 5) The values obtained were comparable to those found in adult rat liver microsomes (GNOSSPELIUS *et al* 1969) It is also apparent from fig 5 that despite the fact that the liver specimens were obtained from foetuses with gestational ages from 14 to 25 weeks, there was no apparent correlation in either content of cytochrome P-450 or activity of NADPH-cytochrome *c* reductase with length of gestation Since neither of these cytochromes appears to be rate-limiting in the overall drug hydroxylation reaction, absence of drug metabolism in the foetal liver might be explained by deficient activity of the flavoprotein which serves to reduce cytochrome P–450 This enzyme, NADPH-cytochrome P-450 reductase, is believed to be the rate limiting step in the oxidation according to investigations performed on animal liver microsomes Activity was present in all seven foetal liver samples in which the assay was carried out and the rate of reduction appeared to be similar to that found in microsomal suspensions from adult rabbit and rat liver Thus it

would appear that the electron transport chain was intact in the foetal liver and should be intrinsically capable of oxidizing drug substrates

Our first hint as to why significant metabolism of aminopyrine and benz pyrine did not occur in foetal liver came from an examination of the spectral change that takese place when oxidizable substrates are added to suspensions of microsomes These changes which represent the binding of the substrate to cytochrome P-450 are of two types I is characterized by a peak at about 390 nm and a trough at about 420 nm and II by the reverse, a trough in the 400 nm region and a peak in the 430 nm region of the difference spectrum of the microsomes Addition of endogenous substrates, such as laurate or testosterone, produced a type I spectral change These two substrates were found in subsequent experiments to be oxidized by foetal liver microsomes at rates comparable to those found in adult rat liver microsomes On the other hand, aminopyrine, which was not significantly demethylated in the microsomes, gave rise to a type II spectral change (fig 6) Aminopyrine normally produces a type I spectral change in animal liver microsomes Thus we can hypothesize that the deficient drug metabolism of the foetus is due to the presence at the catalytic site of the terminal oxidase of endogenous substrates with a high affinity for the site Drug substrates cannot gain entry to the site and thus are not oxidized,

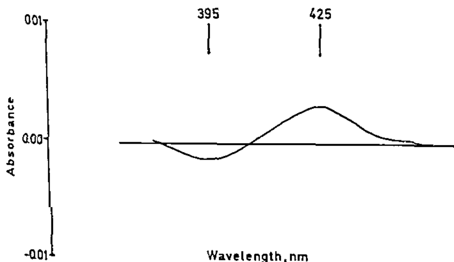


Fig 6 Spectral change produced by the addition of aminopyrine (final concentration 3.3 mM) to a suspension of liver microsomes from a 22 week old foetus Each cuvette contained microsomes corresponding to 4.25 mg of protein in 2 9 ml of TRIS-KCl buffer pH 7.5 (from YAFFE *et al* 1970)

even though all of the components of the microsomal electron transport chain are present in sufficient amounts The increase in drug metabolic activity noted soon after birth may be due to a decrease in concentration of endogenous substrates no longer being furnished by the maternal organism or the placenta Finally, it should be emphasized that the presence of these components in human foetal liver clearly indicates a species difference, since they have not been found in the foetal liver of different animals This serves as yet another example of the need for and importance of pharmacologic studies in man

Acknowledgements

The expert technical assistance of Miss Margareta Sparthan and Mrs Hjordis Thor in the foetal studies (which were performed in collaboration with Dr Sten Orrenius) is gratefully acknowledged This investigation was completed during the tenure of N I H Special Fellowship HD 07109 to S J Y and was also supported in part by grants from the Swedish Medical Research Coucil (project no B70-13x-2471-03) and the National Institutes of Health, Bethesda, Md , USA (GM 13978-05 to Professors B Holmstedt and F Sjoqvist, HD 04287)

REFERENCES

Ackermann, E Die Demethylierung von Aminophenazon und Codein in der Leber des Menschen *Biochem Pharmacol* 1970, 19, 1955

Alvares, A P , G Schilling W Levin, R Kuntzman, L Brand & L C Mark Cytochromes P 450 and b_5 in Human liver microsomes *Clin Pharmacol and Therap* 1969, 10, 655

Assay, L D & R Koch Colistin for treating pseudomonas infections in children In *Antimicrobial agents and chemotherapy* American Society for Microbiology, Ann Arbor, Michigan, p 466, 1962

Axline S G , S J Yaffe & H J Simon Clinical pharmacology of antimicrobials in premature infants *Pediatrics* 1967 39 97

Barnett, H L , K Hare, H McNamara & R Hare Measurement of glomerular filtration rate in premature infants *J Clin Invest* 1948 27 691

Brown, A K & W W Zuelzer Studies on the neonatal development of the glucuronide conjugating system *J Clin Invest* 1958, 37, 332

Cooper, D Y , S Levin, O Rosenthal & R W Estabrook Photochemical action spectrum of the terminal oxidase of mixed function oxidase system *Science* 1965 147 400

Dallner, G , P Siekevitz & G E Palade Biogenesis of endoplasmic reticulum membranes *J Cell Biol* 1966, 30, 97

Ernster, L. & S Orrenius Substrate-induced synthesis of hydroxylating enzyme system of liver microsomes *Fed Proc* 1965, 24, 1190

Fouts, J R & R H Adamson Drug metabolism in the newborn rabbit *Science* 1959 129, 897

Gnosspelius, Y., H Thor & S Orrenius A comparative study of the effects of phenobarbital and 3,4-benzpyrene on the hydroxylating system of rat liver microsomes *Chem. Biol Interactions* 1969, **1**, 125
Jondorf, W R, R. P Maickel & B B Brodie Inability of Newborn mice and guinea pigs to metabolize drugs *Biochem Pharmacol* 1958, **1**, 352
Mirkin, B L. Developmental pharmacology *Ann Rev Pharm* 1970, **10**, 255
Nyhan, W L. & F Lampert Response of the fetus and newborn to drugs *Anesthesiology* 1965, **26**, 487
Pomp, H, M Schnoor & K J Netter Untersuchung uber die Arzneimittel Demethylierung in der Fetalen Leber *Deutsche Medizinsche Wochenschrift* 1969, **94**, 1232
Weiss, C F., A J Glazko & J K. Weston Chloramphenicol in the newborn infant *New England J Med* 1960, **262**, 787
West, J R., H W Smith & H. Chasis Glomerular filtration rate effective renal blood flow, and maximal tubular excretory capacity in infancy *J Pediat* 1948, **32**, 10
Wright, W W & H Welch Chemical, biological, and clinical observations on colistin *Antibiot Ann* 1959-60 **61**, 1960
Yaffe S. J Some aspects of perinatal pharmacology, *Ann Rev Med* 1966, **17**, 213
Yaffe, S J., A. Rane, F Sjoqvist, L. O Boreus & S Orrenius The presence of a monooxygenase system in human fetal liver microsomes *Life Sci* 1970, **9**, 1189

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REFERENCES

Ackermann E Die Demethylierung von Aminophenazon und Codein in der Leber des Menschen *Biochem Pharmacol* 1970 19, 1955

Alvares, A P, G Schilling, W Levin, R Kuntzman, L Brand & L C Mark Cytochromes P 450 and b₅ in Human liver microsomes *Clin Pharmacol and Therap* 1969, 10, 655

Assay, L D & R Koch Colistin for treating pseudomonas infections in children In *Antimicrobial agents and chemotherapy* American Society for Microbiology, Ann Arbor, Michigan, p 466, 1962.

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Ernster, L. & S Orrenius Substrate induced synthesis of hydroxylating enzyme system of liver microsomes *Fed Proc* 1965, 24 1190

Fouts, J R & R H Adamson Drug metabolism in the newborn rabbit *Science* 1959 129, 897

Method

Twenty five pregnant women undergoing legalized abortion for socio-medical reasons volunteered in the study They were all in the first half of the middle trimester of pregnancy An intravenous injection of 1 or 2 g of ampicillin (ampipenin®, KABI) was given before the hysterotomy The time interval between the administration and the operation was varied between 5 minutes and 4½ hours This means that in some cases the injection was made while the patient was anaesthetized and, in some cases, before anaesthesia had begun

The maternal plasma concentration of ampicillin in capillary blood was followed by repeated sampling during the period up to removal of the foetus At that moment, a sample was taken of foetal mixed cord blood or blood from the ascending aorta

The blood samples were collected in heparinized capillary tubes routinely used for haematocrit determination After centrifugation, 10 µl of plasma was applied on a filter paper disc with 5 mm diameter by means of a Beckman micropipette These discs, 2–4 per sample, were then placed on pepton free beef infusion agar with 5 % citrated horse blood inoculated with Sarcina lutea ATTC 9341 The inhibition zone diameters were determined after 12–16 hours in 37° and the corresponding plasma concentrations calculated from the standard curve which was always linear with a correlation coefficient of at least 0 99 Plasma concentrations down to 0.5 µg/ml could usually be reliably determined. A detailed evaluation of the procedures for sampling and assay has been performed (JALLING MALMBORG, LINDMAN & BORÉUS unpublished results) Control foetal plasma from patients which had not received ampicillin gave no inhibition zones

Results

Maternal plasma concentrations Ampicillin elimination followed first order kinetics (fig 1) The linear curve on the semilogarithmic plot, however, was preceded by a non linear distribution phase The half life for the drug was 0 49 ± 0 22 hours (S D), as calculated by computer, and 0 51 ± 0 18 hours (S D) if the curves were fitted by eye

The rate of elimination was not correlated to gestational age (crown rump length of the foetus) Neither the premedication (morphine or oxycodone + scopolamine) nor the anaesthesia (halothane + N_2O + O_2) influenced the slope of the curve

Foetal plasma concentration Ampicillin was found already after 5 minutes following the intravenous injection to the mother However, the concentrations were then low, especially if compared to the maternal levels which were very high at this moment. A relative increase in the foetal/maternal concentration ratio occurred, and following about 90 minutes equal concentrations on both sides of the placenta were reached Thereafter, the foetal plasma levels were higher than those in the mother The concentration ratio was continuously increasing for at least 200 minutes As seen in fig 2, the curve seems to approach a constant ratio of about seven

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Placental Transfer of Ampicillin in Man

By

Lars Olof Boréus

Abstract A pharmacokinetic analysis of the foetal/maternal distribution of ampicillin was performed in 25 pregnant women undergoing therapeutic abortion in the middle trimester of pregnancy The drug was given intravenously before the hysterotomy and the plasma levels were followed during the period up to surgery When the foetus was removed from the uterus a sample of foetal plasma was taken for ampicillin assay By means of varying the time between injection and surgery, the ratio between foetal and maternal plasma levels could be determined at various times following the intravenous administration It was found that the two concentrations became equal after 90 minutes and that the ratio was then steadily increasing for at least 200 minutes If a two compartmental pharmacokinetic analysis is made on the basis of the plasma levels in the mother, it shows that a quasi equilibrium between the central compartment and the tissue compartment is reached already after about 35 minutes following the intravenous injection Thus, the distribution of ampicillin to the human foetus through the placenta is slower than the distribution of the drug to the maternal tissues A placental "barrier" seems to exist in the pharmacokinetic sense

Key words Developmental pharmacology – human foetus – pharmacokinetics – ampicillin – placenta

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The pharmacokinetics of the human foetus is difficult to study experimentally, both for technical and ethical reasons In the clinical situation it is usually not possible to follow continuously the plasma concentration of drugs in both mother and foetus over a sufficient period of time In the present study, advantage has been taken of therapeutic abortions where intact foetuses are available at the operation Even if only one observation of foetal drug concentration can be made for each patient a pharmacokinetic analysis can still be made if the time interval between administration and operation is varied in a series of patients

Method

Twenty five pregnant women undergoing legalized abortion for socio-medical reasons volunteered in the study They were all in the first half of the middle trimester of pregnancy An intravenous injection of 1 or 2 g of ampicillin (ampipenin®, KABI) was given before the hysterotomy The time interval between the administration and the operation was varied between 5 minutes and 4½ hours This means that, in some cases the injection was made while the patient was anaesthetized and, in some cases, before anaesthesia had begun

The maternal plasma concentration of ampicillin in capillary blood was followed by repeated sampling during the period up to removal of the foetus At that moment, a sample was taken of foetal mixed cord blood or blood from the ascending aorta

The blood samples were collected in heparinized capillary tubes routinely used for haematocrit determination After centrifugation 10 µl of plasma was applied on a filter paper disc with 5 mm diameter by means of a Beckman micropipette These discs 2–4 per sample, were then placed on pepton free beef infusion agar with 5 % citrated horse blood inoculated with Sarcina lutea ATTC 9341 The inhibition zone diameters were determined after 12–16 hours in 37° and the corresponding plasma concentrations calculated from the standard curve which was always linear with a correlation coefficient of at least 0 99 Plasma concentrations down to 0 5 µg/ml could usually be reliably determined A detailed evaluation of the procedures for sampling and assay has been performed (JALLING, MALMBORG, LINDMAN & BORÉUS, unpublished results) Control foetal plasma from patients which had not received ampicillin gave no inhibition zones

Results

Maternal plasma concentrations Ampicillin elimination followed first order kinetics (fig. 1) The linear curve on the semilogarithmic plot, however, was preceded by a non-linear distribution phase The half life for the drug was 0 49 ± 0 22 hours (S D), as calculated by computer, and 0 51 ± 0 18 hours (S D) if the curves were fitted by eye

The rate of elimination was not correlated to gestational age (crown rump length of the foetus) Neither the premedication (morphine or oxycodone + scopolamine) nor the anaesthesia (halothane + N_2O + O_2) influenced the slope of the curve

Foetal plasma concentration Ampicillin was found already after 5 minutes following the intravenous injection to the mother However, the concentrations were then low, especially if compared to the maternal levels which were very high at this moment A relative increase in the foetal/maternal concentration ratio occurred, and following about 90 minutes equal concentrations on both sides of the placenta were reached Thereafter, the foetal plasma levels were higher than those in the mother The concentration ratio was continuously increasing for at least 200 minutes As seen in fig 2, the curve seems to approach a constant ratio of about seven

Discussion

The present report on the distribution of ampicillin in the pregnant woman demonstrates that a net transfer of this drug from maternal to foetal plasma occurred during 90 minutes following a single intravenous dose Thereafter, the foetal plasma levels were higher, making a diffusion possible in the reverse direction This is in accord with the theoretical model described by GOLDSTEIN et al (1968) The overshoot in foetal plasma concentration is of limited therapeutic significance since the absolute ampicillin concentrations are then low due to the short half-life of the drug However, in the clinical situation, the repeated administration of the antibiotic would increase the blood levels up to bactericidal values

It is therefore of great interest to analyze the elimination rate of the drug in the foetal plasma and compare it to the rate of decline in the mother It is seen from fig 2 that a tendency was found towards a constant foetal/maternal plasma concentration ratio of about 7 In the present study, the experiment

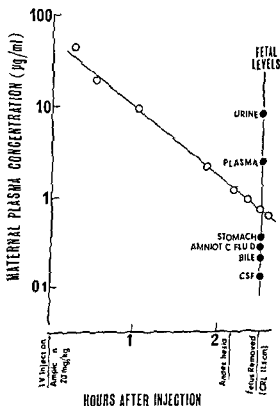


Fig 1 Maternal and foetal ampicillin concentrations following an intravenous injection (20 mg/kg) In this case, the foetus was removed from the uterus 2½ hours after the injection Foetal concentrations in urine stomach contents amniotic fluid bile and cerebrospinal fluid were also determined

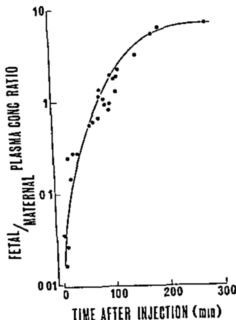


Fig. 2. Foetal/maternal plasma concentration ratio for ampicillin plotted on a logarithmic scale against time after injection.

could not be prolonged beyond the 4½ hour period both for practical reasons and because the maternal ampicillin levels were then so low that they were close to the limit of the sensitivity of the assay method Further experiments with another protocol will therefore be necessary in order to analyze the time period after 4½ hours and to establish if a constant foetal/maternal concentration ratio over a more prolonged period is in fact maintained

If such a constant concentration ratio exists, a value of 7 is unexpectedly high. An interpretation of this finding is not yet possible A difference in protein binding for the drug in maternal and foetal plasma cannot be an explanation since in equilibrium dialysis experiments we have found that ampicillin is much less bound in human foetal than in human maternal plasma (Ehrnebo, Agurell, Jalling & Boréus, to be published in Europ J Clin Pharmacol 1971)

It is interesting to compare the rate of transfer of ampicillin over the placenta with the rate of distribution of the drug in the maternal tissues Such a comparison can be made if the pregnant woman is looked upon as a two-compartmental system with a central compartment (plasma) and a tissue compartment. If the individual maternal concentration curves during the experiment are resolved into two components one steeper "alpha" slope and one less steep "beta" slope, and if the intercepts of these two curves at time

zero are determined, it is possible, knowing the dose given, to calculate the fraction of the dose remaining in the central and tissue compartments at any given time (GIBALDI *et al* 1969) A preliminary computer analysis using this method shows that a "quasi-equilibrium" between the central compartment and the tissue compartment is reached already after about 35 minutes

The time difference between 35 minutes to reach this equilibrium and the much longer period (at least 200 minutes) needed to achieve a constant foetal/maternal plasma concentration ratio may be interpreted as a delay in the placental transfer as compared to tissues in general Thus, a placental "barrier' seems to exist in the pharmacokinetic sense

Acknowledgements

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REFERENCES

Gibaldi, M, R. Nagashima & G Levy Relationship between drug concentration in plasma or serum and amount of drug in the body *J Pharmac Sci* 1969, 58 193–197

Goldstein, A, L Aronow & S M Kalman Principles of drug action Hoeber, New York 1968

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Pharmacokinetics and Biological Effects of Nortriptyline in Man

By

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Early studies of the kinetics of desmethylimipramine and nortriptyline established that patients treated with similar doses of the respective compound developed markedly different steady-state plasma levels (HAMMER *et al* 1967, HAMMER & SJOQVIST 1967, SJOQVIST *et al* 1968) For desmethylimipramine (25 mg t i d) a range between 8 and 295 mμg/ml was observed, and the interindividual variability of nortriptyline plasma levels was of the same order of magnitude (fig 1)

These findings raised the following questions

(1) What are the *pharmacokinetic mechanisms behind these individual* differences, and

(2) what is their significance in terms of drug response

This paper summarizes our efforts to elucidate these two questions The

(c) experimental and clinical studies of the effects of NT in relation to steady state plasma levels.

Methods

A variety of chemical, pharmacological and clinical methods have been used The reader is referred to the original papers for details.

Care has been taken to validate the drug analytical data obtained utilizing recent developments in analytical chemistry *During the early part of the project NT was* measured according to the principles described by HAMMER & BRODIE (1967) for acetylation of secondary amines with ³H acetic anhydride as further described by SJÖQVIST *et al.* (1969b) *Specificity is achieved by a) careful control of the subjects'* (in patients or healthy volunteers) drug therapy with no other drugs liable to interfere in the assay

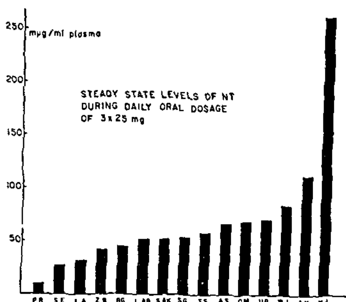


Fig 1 Interindividual differences in steady state plasma concentrations of nortriptyline Each bar is based on 3–5 determinations S D < 10 % From SJOQVIST *et al* (1968)

being administered b) selective extraction and c) thin layer chromatography of the radioactive amide formed (HAMMER & BRODIE 1967, SJOQVIST *et al* 1969b) In parallel experiments NT has been determined with gas liquid chromatography (GLC) utilizing the heptafluorobutyryl derivative and Ciba 34276 as internal standard (BORGÅ & GARLE unpublished results) Good agreement between the two methods has been obtained

Concentrations of NT in plasma in the lower range (< 50 mμg/ml) have also been assayed with mass fragmentography, a technique in which the mass spectrometer is used as a detector (HAMMAR *et al* 1968 & 1969) The fragment 232 in the molecule (HAMMAR *et al* 1971) is used and the internal standard is the same as in the GLC procedure above The specificity of this method is unequivocal (BORGÅ PALMÉR LINNARSSON, SJOQVIST & HOLMSTEDT, unpublished results) Various plasma samples from patients or volunteers who have participated in the subprojects reported below have been analyzed for NT both with the external isotope technique (HAMMER & BRODIE 1967, SJOQVIST *et al* 1969a, b) and mass fragmentography The data obtained with the two methods were found to agree well (BORGÅ PALMÉR LINNARSSON, SJOQVIST & HOLMSTEDT, unpublished results)

Steady-state plasma level

After repetitive oral administration of nortriptyline or desmethylimipramine at fixed intervals the plasma concentration increases successively until an apparent steady-state level is achieved (HAMMER *et al* 1967 HAMMER & SJOQVIST 1967) (fig 2) During steady-state conditions the amount of absorbed drug should be equal to the amount eliminated and the plasma concentration kept almost constant in equilibrium with the body tissues Accord-

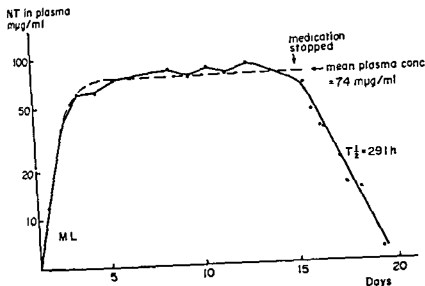


Fig. 2. The plasma concentration of nortriptyline (NT) in a healthy volunteer (M L.) given 0.4 mg nortriptyline orally three times daily for 14 days. From ALEXANDERSON & SJÖQVIST (1971)

ing to WAGNER *et al.* (1965) the steady state plasma concentration (C) of a

the apparent volume of distribution (V_d) and the dosage interval (Δt) as to be expressed mathematically

$$C = 1.44 \frac{F \cdot D \cdot t_{½}}{V_d \cdot \Delta t}$$

Under our experimental conditions the dosage and the dosage interval (8 hours) have been kept constant and F equals 1, since the oral absorption of the tricyclic antidepressants is rapid and almost complete (HAMMER & SJÖQVIST 1969, and unpublished data). Of the two kinetic variables ($t_{½}$ and V_d) controlling C the former depends on active enzymatic processes since most nortriptyline is excreted in the urine as metabolites (SJÖQVIST *et al.* 1969a and unpublished data).

In the studies by HAMMER *et al.* (1967) it was observed that the $t_{½}$ (post steady state) of desmethylimipramine varied between 54 and a few hours in individuals, whose steady-state plasma levels varied by a factor of about 30. These findings suggested that most of the variability in steady state plasma

levels was in fact due to corresponding differences in $t_{1/2}$ and hence in the rate of metabolism of the drug A further explanation of the observed variability would be differences between individuals in V_d

We have hypothesized that the apparent steady state plasma concentration at a given dosage reflects the activity of the enzyme(s) *in the metabolism* *Indirect* support for this view derived from preliminary observations that treatment with phenobarbital (phenemalum NFN), a well-known inducer of drug metabolism, lowered the steady state plasma concentrations of desmethylimipramine (HAMMER *et al* 1967)

Genetic control of the steady state plasma level of nortriptyline and the influence of drug therapy

Pharmacokinetic studies with NT have been performed in healthy twins with a dual purpose (a) to study the role of genetic factors in determining the steady state plasma levels and (b) to assess the influence of concomitant drug therapy on this pharmacokinetic parameter

Seventy-eight identical and fraternal twins, all between 45 and 51 years of age, voluntarily participated in the study, which has been described in detail by ALEXANDERSON *et al* (1969) Nineteen pairs were monozygotic and 20 dizygotic All subjects were given nortriptyline hydrochloride 0 2 mg/kg body weight by mouth three times daily for eight days

In the entire material (fig 3) a 10-fold difference in steady state plasma concentration of NT was obtained (8–78 mμg/ml)

Monozygotic twins, previously not treated with other drugs, achieved practically the same steady state plasma concentration of nortriptyline The individual steady-state levels varied from 14 to 35 mμg/ml plasma (fig 4) The analysis of variance showed no differences in the steady-state plasma

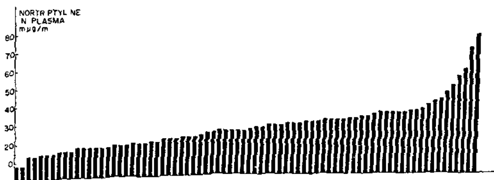


Fig 3 Interindividual differences in steady state plasma concentrations (8 78 mμg/ml) of NT on days 6–8 in healthy twins given 0 2 mg/kg t i d orally for 8 days From ALEXANDERSON *et al* (1969)

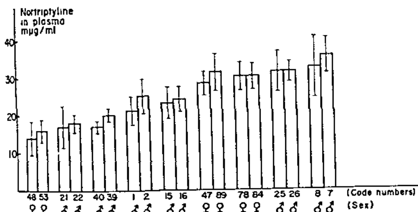


Fig. 4 Mean nortriptyline plasma concentration of days 6, 7 and 8 in identical twins previously not exposed to drugs Data from ALEXANDERSON *et al* (1969)

concentrations between individuals within the pairs but there were significant differences between the pairs ($P < 0.0005$) (ALEXANDERSON *et al* 1969) Dizygotic twins not taking other drugs (fig 5) showed significant intrapair ($P < 0.0005$) and interpair ($0.05 > P > 0.025$) differences in steady-state plasma concentrations of nortriptyline (ALEXANDERSON *et al* 1969)

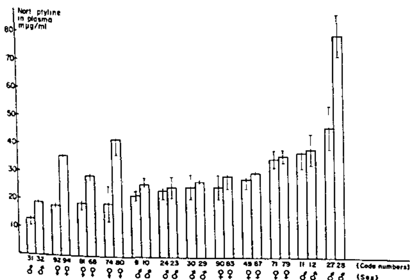


Fig 5 Mean nortriptyline plasma concentration of days 6, 7 and 8 in fraternal twins previously not exposed to drugs Data from ALEXANDERSON *et al* (1969)

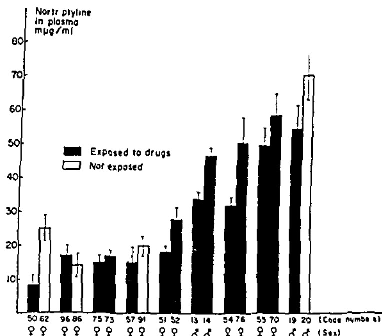


Fig 6 Mean nortriptyline plasma concentration of days 6, 7 and 8 in identical twins exposed to different drugs Data from ALEXANDERSON *et al* (1969)

The intrapair similarity in steady-state plasma concentrations was not found in monozygotic twins who were on treatment with various other drugs, during the experiment (fig 6) The statistical calculations revealed significant differences in steady-state plasma concentrations of nortriptyline both between individuals within pairs ($P < 0\ 0005$) and between the pairs

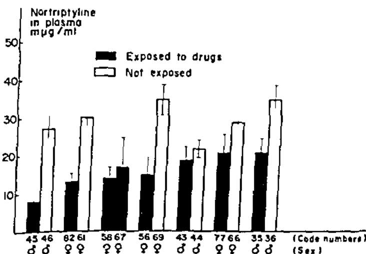


Fig 7 Mean nortriptyline plasma concentration of days 6 7 and 8 in fraternal twins exposed to different drugs Data from ALEXANDERSON *et al* (1969)

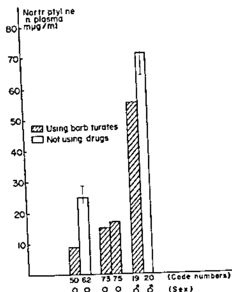


Fig. 8. Steady state plasma concentrations of NT in identical twins, where one or both in the pair were on concomitant treatment with small doses of barbiturates. The NT levels are low (compared to the entire material) in the pair where both twins were on barbiturates. Computed from ALEXANDERSON *et al.* (1969). The drug history available from the author.

($0.01 > P > 0.0005$) (ALEXANDERSON *et al.* 1969). The other drugs were mainly small doses of hypnotics and/or anxiolytics known to induce drug metabolism in animals (ALEXANDERSON *et al.* 1969).

The intrapair variations in steady-state levels of NT were even more pronounced in dizygotic twins exposed to other drugs (fig. 7) probably due to additive influences of both genetic factors and drug therapy (ALEXANDERSON *et al.* 1969).

Fraternal and identical twins treated with various drugs containing barbiturates had considerably lower steady-state plasma concentrations of NT than the untreated control twins (ALEXANDERSON *et al.* 1969) (fig. 8).

There is no evidence that barbiturates or other drugs interfere with the absorption of tricyclic antidepressants or change their volume of distribution. Regarding the latter, it is possibly relevant that phenobarbital does not affect the binding of desipramine to plasma proteins (BORGÅ *et al.* 1969). These facts suggest that the steady-state level of nortriptyline is decreased in barbiturate-treated twins owing to induction of microsomal drug metabolizing enzymes.

From the studies above it was concluded that most of the variability in

NT steady-state plasma concentrations between persons who have not received drugs is genetically determined Exposure to other drugs also influences the steady-state plasma concentration of NT, which in a given patient may therefore be determined by a resultant of genetic and environmental factors

Family studies have been performed by ASBERG et al (1971b) in order to further elucidate the mode of inheritance of NT steady state plasma levels Early studies indicated an apparent bimodality in the distribution of NT plasma levels in psychiatric patients, which might be an indication of a genetic polymorphism, where patients developing extremely high plasma levels would represent rare recessives (HAMMER & SJOQVIST 1967, SJOQVIST et al 1968) 29 relatives of three patient propositi with extremely high plasma levels were compared to twenty random subjects All subjects were given NT orally 25 mg t i d There was no tendency for NT plasma levels to fall into two modes in the relatives or in the entire material but mean and variance of values were larger in the relatives than in the ran

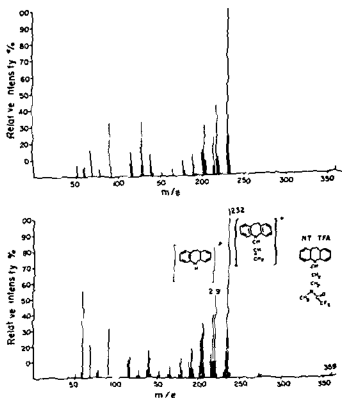


Fig 9 Mass spectrum of a compound (upper panel) in a trifluoroacetylated extract of a plasma from a patient treated with nortriptyline (NT) The compound had the same GLC retention time as synthetic NT treated with trifluoroacetic anhydride (TFAA) and the same mass spectrum as NT TFA (lower panel) After HAMMAR et al (1971) By kind permission of the publisher

dom subjects (ASBERG *et al* 1971b) The apparent bimodality of the distribution in patients might be explained by the extreme skewness of the distribution and the fact that the patients had been previously treated with other psychotropic drugs (HAMMER & SJOQVIST 1967, SJOQVIST *et al* 1968).

The mode of inheritance of NT steady-state plasma levels is likely to be polygenic, i e controlled by an unknown number of allelic genes (ASBERG *et al* 1971b) This could be in agreement with the findings of individual variations in both plasma half-life and apparent volume of distribution of NT (see below)

Metabolism of nortriptyline

Three previously unidentified metabolites of NT have been found in body fluids of man with the aid of gas chromatography-mass spectrometry

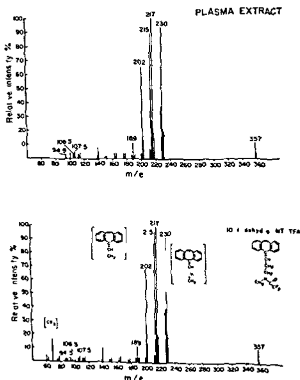


Fig 10 Mass spectrum of a metabolite of NT after derivative formation with TFAA (upper panel) The metabolite had the same retention time and mass spectrum (lower panel) as synthetic 10,11-dehydro-NT TFA. The true nature of the metabolite was found to be 10-hydroxy NT that loses water during derivative formation (HAMMAR *et al* 1971) By kind permission of the publisher

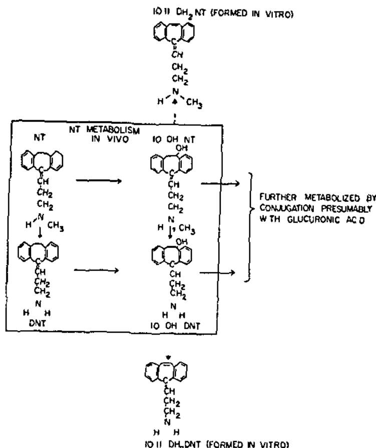


Fig 11 Metabolism of nortriptyline in man as ascertained with gas chromatography mass spectrometry

(HAMMAR *et al* 1971) Conclusive evidence was obtained that NT is hydroxylated in the 10-position and demethylated in the side chain Tentative identification of the metabolite 10 hydroxydesmethylnortriptyline (10-OH-DNT) was also achieved This research was complicated by the phenomenon that 10-hydroxynortriptyline (10 OH-NT) was dehydrated during derivative formation with trifluoroacetic anhydride (TFAA) and thus showed up (figs 9–10) as 10,11-dehydro-NT-TFA (HAMMAR *et al* 1971) The results of this work are summarized in fig 11

Quantitative studies on the relative importance of the various pathways are in progress 10-OH-NT seems to occur in plasma in relatively low concentrations, although GLC studies of urine (BORGÅ *et al*, unpublished results) show that 10-OH-NT is the main metabolite of NT Presumably 10-OH-NT is rapidly conjugated with glucuronic acid and quickly excreted by the kidney Perhaps hydroxylation is the rate limiting step in the metabolism of NT in man This hydroxylation is stereospecific and the cis and trans isomers of 10 OH-NT have been separated and quantitated

in urine from human volunteers treated with NT (BERTILSSON & ALEXANDERSON, unpublished results)

Desmethylated nortriptyline (DNT) seems to accumulate to very small concentrations in human plasma compared to NT This is of interest in relation to the observations by GILLETTE (1963), that rat liver microsomes contain an enzyme which readily demethylates the tertiary amines imipramine and amitriptyline but demethylates the secondary amine desmethylimipramine much more slowly DNT seems to occur in rather constant proportions (5–10 %) of NT (HAMMAR *et al* 1971, and unpublished results) These data also explain the small interference of DNT in the assay of NT in plasma with the Hammer Brodie procedure

Studies are also in progress to relate the plasma half-life of NT after repetetive or single oral doses to the metabolite excretion in the urine (ALEXANDERSON & BORGÅ, unpublished results) These studies show that the excretion rate of 10 OH NT parallells the disappearance rate (so called β-slope) of NT from plasma Interindividual differences in $t_{1/2}$ of a single oral dose thus correlate to the rate of excretion of 10 OH-NT in the urine

Predictability of the steady-state plasma concentration of NT from single oral dose experiments

These studies aim at elucidating the relative importance of plasma $t_{1/2}$ of a single oral dose of NT (i e half time of elimination from plasma after apparent distribution equilibrium) and its apparent volume of distribution (V_d) for the steady-state plasma concentration obtained on repetetive treatment with NT (14 days) ALEXANDERSON & SJOQVIST (1971) have shown that the steady-state plasma levels of NT are correlated to the ratio between $t_{1/2}$ and V_d as calculated in the same individuals from single oral dose experiments (fig 12) This tentative possibility to calculate an optimal steady state plasma level of NT prior to long term treatment will be further investigated in patients

Binding and distribution of nortriptyline (and other tricyclic antidepressants)

We have also studied the binding of various tricyclic antidepressants to human plasma (*in vitro*) and the effects thereupon of other drugs Heparinized human plasma was used rather than albumin because it is not known to which molecular species these drugs are bound An ultrafiltration method and labelled compounds were utilized (BORGÅ *et al* 1969) At a total concentration of 0 29 μg/ml and at room temperature the percentage of unbound desmethylimipramine (DMI) was found to be 9 5 ± 1 4 in 41

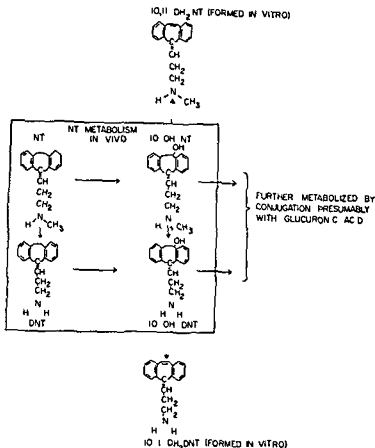


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individuals (BORGÅ *et al* 1969), suggesting small interindividual differences

The unbound fraction of DMI in plasma increased only twofold when the total concentration of the drug was increased a thousand times The degree of binding over the entire range of therapeutic plasma drug concentrations was relatively constant Nortriptyline was bound to 5 5 ± 0 6 per cent The binding for all investigated compounds was slightly lower at 37° Displacement effects of other drugs (acidic and basic) seem to be of little importance (BORGÅ *et al* 1969)

The data on NT-binding *in vitro* agree well with our finding *in vivo* that under steady-state conditions the concentration of NT in cerebrospinal fluid (CSF) averages 7 per cent of the plasma level (BORGÅ *et al* 1969)

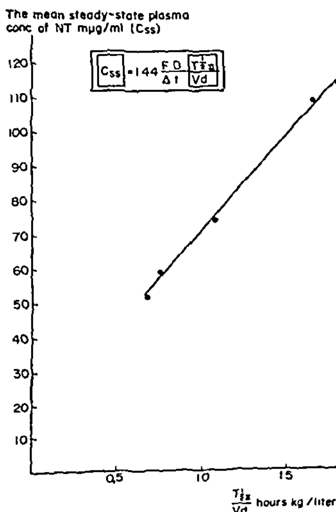


Fig 12 Correlation between the found NT steady state plasma concentration (y axis) in healthy humans treated with NT for 14 days and the ratio between plasma half life ($t_{1/2}$) and the apparent V_d (x axis) calculated in the same individuals after a single oral dose of NT From ALEXANDERSON & SJÖQVIST (1971)

At equilibrium the drug concentration in CSF (which can be considered to be practically free of protein) should be equal to the unbound concentration in plasma

The biological significance of the plasma protein binding of nortriptyline has been assessed by comparing its inhibitory effect on the uptake of noradrenaline (NA) in adrenergic nerves from the rat's iris incubated in salt buffer or human plasma (BORGÅ *et al* 1970) The procedure takes advantage of the primary action of tricyclic antidepressants, i e to inhibit the neuronal uptake of monoamines

When added to the incubation medium, NT inhibited the uptake of NA approximately ten times as effectively in buffer as in human plasma (fig 13) within the tested concentration range of NT (10^{-8}–10^{-6}M) The concentration of NA used (10^{-7}M) is thought to be close to the physiological one Thus this bioassay procedure indicates that NT is bound to about 90 % (at 37°) The much weaker inhibitory effect of NT on NA uptake in nerves incubated in plasma compared to buffer was easily shown in the fluoresence microscope utilizing the histochemical method for demonstrating noradrenaline (BORGÅ *et al* 1970)

The experimental design also afforded an opportunity to investigate whether the uptake of NA was inhibited in adrenergic nerves incubated in plasma from patients under treatment with recommended doses of NT Plasma from patients treated with NT was indeed found to inhibit NA uptake in the iris and a correlation was established between the inhibitory

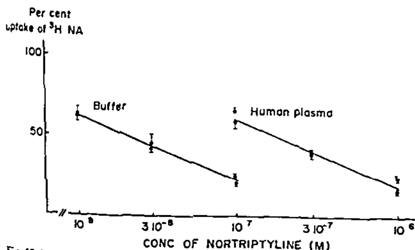


Fig. 13. The inhibitory effect of various concentrations of NT on the uptake of ^{3}H NA in isolated irides from untreated rats incubated in 5 ml of Krebs-Ringer buffer or human plasma. The uptake of ^{3}H NA is expressed as per cent of controls. Each point represents the mean ± standard error of 8 irides. From BORGÅ *et al* (1970) by kind permission of the publisher

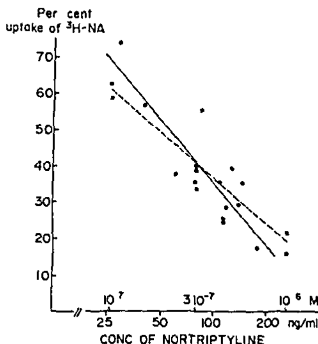


Fig 14 Neuronal uptake of ^{3}H noradrenaline (expressed as per cent of control) in irides incubated in plasma from patients (●) treated with nortriptyline in relation to "endogenous" nortriptyline plasma concentrations. Solid line represents regression line (coefficient of correlation = 0 83, $P < 0\,001$) for 14 patients. Dashed line represents the regression line (coefficient of correlation = 0 98, $P < 0\,001$) for model experiments (■) in which nortriptyline was added to blood donor plasma From Borgå *et al* (1970) by kind permission of the publisher

effect and the "endogenous' plasma level of NT (fig 14) Since the bioassay measures the overall effect on the adrenergic neuron of NT and its metabolites in plasma the correlation obtained favours the view that NT *per se* accounts for the pharmacologic effects This is further supported by the following experiment When NT was added in different concentrations to control plasma the inhibitory effect observed on NA uptake was close to that obtained with patient plasma containing the same "endogenous" concentration of NT (fig 14)

In further experiments the specificity of the rat iris test in relation to thymoleptic and neuroleptic drugs has been investigated (Tuck, Hamberger & Sjöqvist, unpublished results) These studies indicate that only plasma drawn from patients being treated with thymoleptic drugs inhibit NA uptake under the experimental conditions Confirmatory results have been obtained when the rat's iris was replaced by slices of cerebral cortex

Unequivocal data on the apparent volume of distribution (V_d) of NT would require experiments with intravenous administration of doses too high to be adviceable However, indirect assessment of V_d is possible from oral experiments for drugs like NT which have a fast absorption

rate compared to the elimination rate (WAGNER *et al.* 1965) These calculations reveal a two fold variation between individuals in V_d (ALEXANDERSON & BORGÅ, unpublished results) The values are in the order 15–30 l/kg The extensive tissue distribution of NT includes the human foetus We have recently studied a pregnant woman who attempted suicide with an overdose of NT one day before delivery The kinetic data obtained in the mother and the newborn child suggest that NT is rapidly distributed through the placental barrier (SJOQVIST *et al* 1971)

The overall data on the binding and distribution of NT indicate that interindividual variations in these parameters are small compared to the interindividual variations in metabolism

Correlation between the steady state plasma level of NT and its pharmacologic effects in man

For model studies of this problem we have used tyramine as a tool drug. Reasonable evidence had been obtained that the parent compound

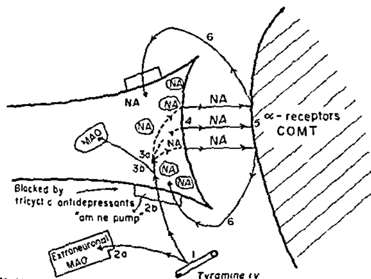
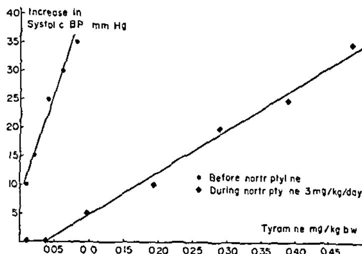


Fig. 15 Model showing the fate and action of intravenously injected tyramine (after FRISCHOLSS *et al* 1970) NA = noradrenaline, MAO = monoamine oxidase, COMT = catechol-O-methyl transferase
1 = intravenous injection of tyramine (TA)
2a = metabolism of TA through extraneuronal MAO
2b = active uptake of TA through the axonal membrane.
3a = TA displaces NA
3b = metabolism of TA by intraneuronal MAO
4-5 = release of NA onto α receptors, pressor effect
6 = reuptake of NA.



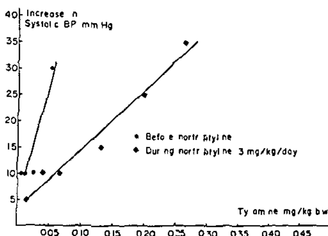


Fig 16a, b Relation between systolic pressor effect and dose of tyramine before and during treatment of two patients with NT (3 mg/kg/day for about 3 weeks)
16a = patient B in fig 17
16b = patient F in fig 17

NT rather than its metabolites was pharmacologically active (see above) The rational behind the use of tyramine in these experiments has been discussed in detail in a previous paper (FREYSCHUSS *et al* 1970) Tyramine is injected intravenously and the short-lasting systolic pressor effects recorded before and during treatment with NT Tyramine probably evokes its action by displacing intraneuronal noradrenaline (fig 15) Tricyclic antidepressants antagonize the pressor effect of tyramine by inhibiting its active uptake into the neuron (for references see FREYSCHUSS *et al* 1970) At higher doses they may also antagonize the effects of the released noradrenaline on the alpha-adrenergic receptors (FREYSCHUSS *et al* 1970, SIWERS *et al* 1971)

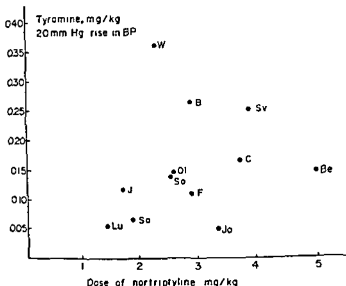


Fig. 17 Relationship between dose of nortriptyline (mg/kg) and the increase in dose of tyramine (mg/kg) producing a 20 mm Hg rise in systolic blood pressure No correlation ($r = 0.18$, $0.6 > P > 0.5$) From FREYSCHUSS *et al* (1970)

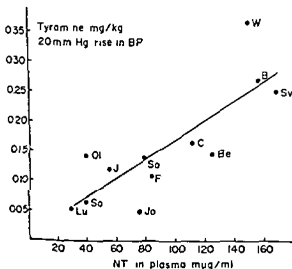


Fig. 18. Correlation between plasma level of nortriptyline and blockade of tyramine pressor effects ($y = 0.016\,x + 0.0084$, $r = 0.81$ $P < 0.005$) The data are the same as in fig. 17 From FREYSCHUSS *et al* (1970)

Systolic pressor effects after intravenous injection of tyramine were recorded in 13 patients before and during treatment with NT in doses of 2 4–5 mg/kg/day for 3–4 weeks During nortriptyline treatment a several-fold decrease in the responsiveness to tyramine occurred Patients treated *with the same dose of NT* showed markedly different decreases in the responsiveness to tyramine (fig 16) There was no correlation between the blockade of the tyramine pressor effects and the dose of NT given (fig 17) By contrast a significant correlation was obtained between the blockade and the steady state plasma concentration of NT (fig 18) Thus the individual differences in response to NT *are of a much lower magnitude* when the drug s action is related to an estimate of the biological availability of the compound rather than to the dose administered (these in patients were observed to ingest their medicine) Tyramine pressor effects were not changed in a small control group of depressed patients after electro convulsive treatment (FREYSCHUSS *et al* 1970)

It should be noted that a few patients in fig 18 such as W do not fit the line Other mechanisms of inter individual differences in the response to antidepressants may operate but the only way to unravel these is to monitor the kinetics of the compounds We have recently come across a rather unique patient who tolerated 0 5 g of imipramine daily although her plasma level of the metabolite desmethylimipramine was incredibly high, namely 950 mμg/ml There are at present few well documented examples in the pharmacological literature of genetically determined ab normalities in receptor site sensitivity the hereditary resistance to cou marin analogues being a notable exception (O REILLY *et al* 1968)

Correlation between the steady state plasma concentration of nortriptyline and its clinical effects

The bearing of the individual variations in pharmacokinetics of nortrip tyline on the occurrence of subjective side effects has been investigated by ASBERG *et al* (1970) in 40 psychiatric in patients with depressive disorders Plasma levels were determined before and during 4 weeks of treatment with NT 50 mg t i d and patients were rated (fig 19) for subjective side effects, the raters being unaware of the plasma levels of the drug The percentage of patients who had any of the 11 rated side effects" were similar during treatment with placebo and NT but the severity of symptoms were more pronounced during NT therapy Controlled" studies using graded rating scales of side effects therefore appear essential in this type of research

In most cases the side effects of NT were rather moderate In two cases there were however more alarming side effects In one of these a

Fig. 19

Item	Score
1 *Physical tiredness*	
None	0
Slightly tired but needs no extra rest	1
Has to lie down and rest at times	2
Lies all day	3
2. *Sleep disturbance*	
Normal sleep without hypnotics	0
Normal sleep with hypnotics	1
3–6 hours sleep with hypnotics	2
Less than 3 hours sleep with hypnotics	3
3 *Headache*	
None	0
Occasional no analgesics needed	1
Constant moderate, or occasional severe headache. Salicylates effective	2
Constant severe headache Salicylates ineffective	3
4 *Vertigo*	
None	0
Occasional slight dizziness	1
Constantly slightly dizzy or occasional severe dizziness	2
Constant severe dizziness Has to lie down	3
5 "*Orthostatic*" *symtoms*	
None	0
Feels slightly like fainting on sudden rising	1
Has to rise slowly to avoid feeling like fainting	2
Fainting	3
6 *Palpitations*	
None	0
Slight palpitations	1
Occasional disturbing palpitations	2
Constant disturbing palpitations	3
7 *Tremor*	
None	0
Slight tremor movements not affected	1
Obvious tremor, small movements impaired	2
Severe tremor	3
8 *Perspiration*	
Normal	0
Slightly increased	1
Obviously increased	2
Profuse	3
9 *Dryness of mouth*	
None	0
Some, but not subjectively disturbing	1
Obvious but not severe or painful	2
Severe makes speaking difficult	3
10 *Constipation*	
None	0
Slight constipation purgatives not needed	1
Obvious constipation Purgatives taken	2
No bowel movements in spite of purgatives	3
11 *Micturition disturbances*	
None	0
Slight difficulties in passing water	1
Difficulties in emptying bladder Treatment needed	2
Urinary retention	3

Fig. 19 Rating scale for side-effects of nortriptyline (from ASBERG CRONHOLM, SJÖQVIST & TUCK 1970)

44 years old woman, the dosage had to be reduced because the patient fainted Another patient, a 51 year old man developed a right bundle branch block during ECG exercise test, which disappeared after withdrawal of NT (ASBERG *et al* 1970) These two patients had the highest plasma levels of NT in the material (340 and 235 mµg/ml respectively)

There was a large variation in plasma levels between individual patients, but in any given patient the plasma level tended to be constant over a

*Table 1**

A *Regression of corrected side effect scores (y) on plasma level of nortriptyline (x)*

	n	b	r	t	level of significance
Week no 1	35	0 04	0 50	3 3558	$P < 0\ 005$
Week no 2	39	0 03	0 46	3 1407	$P < 0\ 005$
Week no 3	36	0 03	0 42	2.7339	$P < 0\ 01$
Week no 4	26	0 01	0 12	0 5899	N S

B *Regression of corrected side effect scores (y) on dosage of nortriptyline in mg/kg body weight (x)*

	n	b	r	t	level of significance
Week no 1	38	0 99	0 14	0 8576	N S
Week no 2	39	0 89	0 12	0 7507	N S
Week no 3	36	−0 05	−0 01	−0 0349	N S
Week no 4	28	1 40	−0 18	−0 9158	N S

n = number of patients (i e pairs of observations)
b = regression coefficient
r = correlation coefficient
N S = not significant
* Quoted from ÅSBERG, CRONHOLM, SJÖQVIST & TUCK, (1970)

period of time The side-effects of nortriptyline diminished significantly with time and were in most cases absent during the fourth week of treatment

There was a significant positive correlation between plasma level of nortriptyline and subjective side-effects (table 1)

The association between subjective side effects and a high plasma level of NT has been confirmed in healthy volunteers, receiving 25 mg of NT three times daily for eight days (ÅSBERG et al 1971b) These data validate the impression from an uncontrolled study (SJÖQVIST et al 1968) that the side-effects of this class of compounds are related to the plasma level

The relationship between plasma level of NT and its antidepressant effect has been studied in 32 in-patients, 10 men (mean age 42 years) and 22 women (mean age 55 years) (ÅSBERG et al 1971a) All patients were diagnosed as "endogenously" depressed Endogenous depression was defined as a disease characterized by the following features (1) lowering of mood, usually without obvious psychological cause, (2) retardation, (3) diurnal variation of symptoms with amelioration in the afternoon, and (4) early awakening, in accordance with the description of CRONHOLM & OTTOSSON (1960)

Patients were rated for severity of depression during the initial placebo period and once weekly during active therapy on a rating scale designed by CRONHOLM & OTTOSSON (1960) The rating was performed by two psychiatrists in a joint interview The raters were kept unaware of the plasma concentrations of nortriptyline until the study was finished The difference between depression score during placebo and active treatment ("amelioration score") was used to estimate therapeutic effect (ÅSBERG et al 1971a)

The amelioration scores for patients in different plasma level intervals after two weeks of treatment are shown in table 2 For the three plasma level classes "low" ($\leq$ 49 mμg/ml), "intermediate" (50–139 mμg/ml), and high ($\geq$ 140 mμg/ml), there is a significant difference in therapeutic effect between the groups ($X^2 = 10\ 89$, df = 2, $P < 0\ 01$) The antidepressant effect is poor at a low plasma level, then increases and finally decreases at very high plasma concentrations

A curved relationship between plasma level of nortriptyline and therapeutic effect, with less response to both extremely low and extremely high plasma levels of the drug, could be explained on the basis of the monoamine hypothesis of depressive disorders It is generally assumed that the therapeutic action of the tricyclic antidepressants is related to their capacity of inhibiting reuptake of transmitter substances in central monoaminergic neurons, thereby increasing the amount of monoamine available at the receptor (CARLSSON 1965, SCHILDKRAUT & KETY 1967) In animal studies, however, these drugs have a dual action HAEFELY et al (1964) showed

gic effects by a variety of tricyclic

and desmethylimipramine, disap-

[illegible] This is probably due to a blockade of the adrenergic receptor, similar to that seen with phenothiazine compounds (OSBORNE & SIGG 1960) Probably this latter action will tend to antagonize the primary effect on the monoamine neurons

Table 2

Mean and standard error of amelioration score for NT plasma concentration intervals (from ÅSBERG, CRONHOLM, SJÖQVIST & TUCK, 1971a)

NT plasma conc interval, mμg/ml	Number of patients	Amelioration score Mean ± S E M
≤ 49	5	0 4 ± 1 2
50– 79	10	6 2 ± 0 8
80–109	4	6 1 ± 1 4
110–139	5	5 0 ± 2.0
≥ 140	5	1.2 ± 1 9

Since very high plasma levels give rise to more side-effects and the therapeutic effect diminishes, the conventional strategy of raising the dosage when the patient does not respond to the drug may often prove unsuccessful in the case of NT

Pharmacokinetic cross-over experiments with nortriptyline and desmethylimipramine

Cross-over experiments have been performed in five psychiatric patients who were treated with desmethylimipramine (DMI) for three weeks followed by NT during three weeks and DMI for further three weeks (HAMMER et al 1969) The steady-state plasma levels of DMI were reproducable at the second treatment period in each individual but varied five-fold between individuals A corresponding variability was found in NT steady-state concentrations, individuals who had low plasma levels of DMI had low steady-state concentrations of NT and individuals who had high DMI-levels subsequently developed high NT-levels (HAMMER et al 1969) This finding was confirmed in three further patients (SJOQVIST 1968) Data from these 8 patients are shown in fig 20 The correlation coefficient between the steady-state plasma levels achieved on the two drugs is 0 95

These results were obtained in more or less chronically drug treated individuals It was therefore thought of interest to repeat the cross-over in healthy volunteers Such a study has now been completed Six healthy volunteers have taken single oral doses of NT and DMI respectively and subse

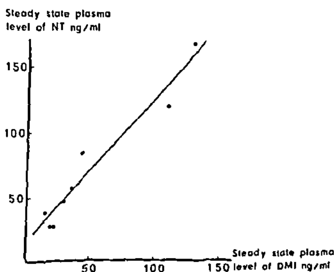


Fig 20 Cross over studies with NT and DMI in eight patients (compare text) The steady state levels obtained on treatment with the respective compound are correlated ($P < 0\,001$, $r = 0\,95$) Data from HAMMER et al (1969) and SJÖQVIST (1968)

quently NT and DMI for 14 days until steady-state The half-lives of DMI and NT after single oral doses were found to correlate well This study will be published shortly. HAMMER *et al* (1969) hypothesized that the steady-state levels obtained with the two drugs in different individuals were correlated because they were metabolized by a common enzyme system which did not distinguish between aromatic (DMI) and alifatic hydroxylation (NT), processes which might be rate limiting in the elimination of the drugs It is probably relevant in this context that both DMI and NT have been found to have extremely high affinities for the so called type I-binding sites on cytochrome P-450 (VON BAHR & ORRENIUS 1971 and unpublished results)

General Discussion

This paper almost exclusively deals with the clinical pharmacokinetics of nortriptyline but the research strategy which has been described should be generally applicable on a vast number of drugs characterized by extensive metabolism in the body and a large volume of distribution

The initial findings of remarkable interindividual differences in the steady-state plasma concentration of tricyclic antidepressant drugs in man (HAMMER *et al* 1967, HAMMER & SJOQVIST 1967, SJOQVIST *et al* 1968) have been confirmed in several studies during the last years (BORGÅ *et al*, unpublished results, ALEXANDERSON *et al* 1969, ÅSBERG *et al* 1971a,b)

The repeatability of the steady-state plasma level of this class of compounds (HAMMER *et al* 1967 & 1969) made it a suitable kinetic parameter in the twin and family studies described above (ALEXANDERSON *et al* 1969, ÅSBERG *et al* 1971a,b,) These investigations ascertained that genetic factors as well as simultaneous treatment with other drugs influence the steady state plasma concentration

The classical pharmacogenetic work on the acetylation polymorphism of isoniazid in man included not only studies of the kinetics of the parent compound in plasma but also quantification of the metabolites in urine and studies of isoniazid-metabolism *in vitro* using human liver biopsy material (PRICE EVANS 1968)

The complex problems regarding the mechanisms of the genetic control of drug hydroxylation in man have to be elucidated similarly by studies of drug metabolite patterns The likely polygenic control of drug hydroxylation (see above) will however render such studies complicated

treatment with phenobarbital due to its lowering effect on NT plasma level, while a patient with very high levels may improve and experience less side-effects by the same treatment All these phenoma will go on without the clinician's knowledge

It appears unequivocal that for patients who do not respond satisfactorily on conventional dosages of NT, monitoring the plasma levels might be rewarding A method of calculating the dosage required for an individual to reach a therapeutic plasma concentration is presently being evaluated in this laboratory (fig 12)

A more general conclusion might be drawn from the experiments The steady plasma level of a drug is probably a more important determinant for its effect than dosage, since it reflects the amount of drug available for biological action Such a conclusion is not valid when the drug is bio-activated in the body, nor when it has an 'irreversible" action such as MAO-inhibitors

It seems justified to state that too little attention is being paid on the pharmacokinetics in man of psychoactive drugs There is a great need for such studies since clinical titration of their dosage is hampered or made impossible by the lack of reliable parameters for quantitative evaluation of the effects in an individual patient Studies of the relationship between kinetics and pharmacologic effects of thymoleptic or neuroleptic drugs are required for a scientfically sound drug therapy It appears much more important to learn about interindividual variations in the kinetics of such drugs than to introduce new compounds

If new drugs are to be introduced, however, drug control agencies should make sure that their kinetics has been properly evaluated and the therapeutic range of plasma concentrations should be known This type of research has previously been difficult due to the lack of analytical techniques sensitive in the nanogram range Also, such studies have been looked upon with scepticism because "so little drug" is available in the plasma pool compared to the tissues, the belief being that the tissue level is more important than the plasma level (forgetting that they are presumably in equilibrium after some time) An excellent example of how rewarding such studies might be is the recent clarification of the relationship between digoxin plasma levels and its cardiac effects – the difference between toxic and therapeutic level amounts to 1 nanogram/ml serum At steady-state, plasma- and heart-levels correlate well (SMITH *et al* 1969) Presumably this research will greatly facilitate a safe and effective use of this 200 years old drug which so far has been on the top list in side effect reports

The question "too little or too much drug" is a universal one in clinical pharmacology, applicable to the entire field of medicine but perhaps it remains unanswered most frequently in psychiatry Much more research is

thus needed to institute an effective yet safe drug therapy is psychiatry This is a great challenge to clinical pharmacology

Acknowledgements

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REFERENCES

Alexanderson, B & F Sjoqvist Individual differences in the pharmacokinetics of monomethylated tricyclic antidepressants Role of genetic and environmental factors and clinical importance *Ann N Y Acad Sci* 1971, **179**, 739–751

Alexanderson, B, F Sjoqvist & D A Price Evans Steady state plasma levels of nortriptyline in twins Influence of genetic factors and drug therapy *Brit Med J* 1969, **4**, 764–768

von Bahr, C & S Orrenius Spectral studies on the interaction of imipramine and some of its oxidized metabolites with rat liver microsomes *Xenobiotica* 1971 **1**, 69–78

Borgå, O, D L Azarnoff, G Plym Forshell & F Sjoqvist Plasma protein binding of tricyclic antidepressants in man *Biochem Pharmacol* 1969, **18**, 2135–2143

Borgå, O, B Hamberger, T Malmfors & F Sjoqvist The role of plasma protein binding in the inhibitory effect of nortriptyline on the neuronal uptake of nor epinephrine *Clin Pharmacol Therap* 1970, **11**, 581–588

Carlsson, A Drugs which block the storage of 5 hydroxytryptamine and related amines In *Handbuch der Experimentellen Pharmakologie* Ed V Erspamer Springer Verlag, *Berlin-Gottingen-Heidelberg 1965*, **19**, 529–592

Cronholm, B & J O Ottosson Experimental studies of the therapeutic action of electroconvulsive therapy in endogenous depression *Acta Psychiat Scand.* 1960, **35**, suppl 145, 69–97

Freyschuss, U, F Sjoqvist & D Tuck Tyramine pressor effects in man before and during treatment with nortriptyline or ECT Correlation between pharmacokinetics and effect of nortriptyline. *Eur J Clin Pharmacol* 1970, **2**, 72–78

Gillette, J R. Metabolism of drugs and other foreign compounds by enzymatic mechanism. In *Progress in drug research* 1963, **6**, 16 Ed. E Jucker Birkhauser Verlag Basel and Stuttgart.

Haefely W, A Hurlimann & H Thoesen Scheinbar paradoxe Beeinflussung von peripheren Noradrenalinwirkungen durch einige Thymoleptika. *Helv Physiol Acta* 1964, **22**, 15–33

Hammar, C-G, B Alexanderson, B Holmstedt & F Sjoqvist. Gas chromatography – mass spectrometry of nortriptyline in body fluids of man – Identification of desmethylnortriptyline and 10-hydroxynortriptyline as metabolites. *Clin Pharmacol Therap* 1971, **12**, 496–505

Hammar, C-G, B Holmstedt, J E Lindgren & R Tham The combination of gas chromatography and mass spectrometry in the identification of drugs and metabolites. *Adv in pharmacology and chemother* 1969, **7**, 53–89

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Plasma Levels of Digoxin in Relation to Toxicity

By

Åke Bertler and Arne Redfors

In earlier studies about the fate of digitalis glycosides in the human body administration of isotope labelled substances have been used In this way many fundamental data about the pharmacokinetics of some heart glycosides have been obtained (DOHERTY 1968) For more extensive clinical investigations, however, this type of technique is not suitable Nor is it useful in the more acute clinical situation, i a in the diagnosis of suspected digitalis intoxications Until now there has been a lack of reliable methods for determination of these drugs in patient blood In the case of digitoxin or other glycosides which are extensively bound to plasma proteins, relatively high plasma levels in the range of 10–40 ng/ml can be achieved after administration of therapeutic doses For an adequate evaluation of such results, however, a separation into bound and free fraction of the drug is desirable The main methodological problem with cardiac glycosides, which are only partially bound to plasma proteins, has been the very low circulating concentration of the drugs, because of their rapid disappearance from plasma For digoxin, given in therapeutic doses, values between 0 and 5 ng/ml have been reported (BINNION *et al* 1969, GRAHAME SMITH & EVEREST 1969, LOWENSTEIN & CORRILL 1966, MARCUS *et al* 1966, SMITH *et al* 1969)

Today two alternative methods for estimation of plasma levels of digoxin seem to be available One is the very specific and sensitive radioimmunoassay, presented by SMITH *et al* (1969), with a lower limit of sensitivity at 0 2 ng/ml plasma. The other method, the principle of which is based on the ability of heart glycosides to inhibit the transport of ^{86}Rb into erythrocytes (SCHATZMANN 1953, LOVE & BURCH 1953), was first described by LOWENSTEIN & CORRILL in 1966 Several investigators have, with varying success, tried to use this method for clinical purposes (BINNION *et al* 1969, COLTER *et al* 1968, GRAHAME-SMITH & EVEREST 1969, LYON & DE GRAFF 1967) When some years ago we started to attack some pharmacokinetical problems on digoxin, we found very soon, like others (BINNION *et al* 1970, EVERED 1970, WILSON 1969) that the original ^{86}Rb method had a low degree of

chlorpromazine and its metabolites in human blood by a new method *Anal Biochem* 1968 25, 532–548

Hammer, W & B B Brodie Application of isotope derivative technique to assay of secondary amines Estimation of desipramine by acetylation with H³-acetic anhydride *J Pharmacol Exp Ther* 1967, 157, 503–507

Hammer, W & F Sjöqvist Plasma levels of monomethylated tricyclic antidepressants during treatment with imipramine like compounds. *Life Sci* 1967, 6, 1895–1903

Hammer, W, C-M Idestrom & F Sjoqvist Chemical control of antidepressant drug therapy In *Proceedings First Internat Symp on Antidepressant Drugs* Milan 1966 Excepta Medica Internat Congr Ser, 1967, 122, 301–310

Hammer, W, S Mårtens & F Sjoqvist A comparative study of the rate of metabolism of desmethylimipramine, nortriptyline and oxyphenylbutazone in man *Clin Pharmacol Ther* 1969, 10, 44–49

O Reilly, R A, J G Pool & P M Aggeler Hereditary resistance to coumarin anti coagulant drugs in man and rat *Ann N Y Acad Sci* 1968, 151, 913–931

Osborne, M & E B Sigg Effects of imipramine on the peripheral autonomic system *Arch int Pharmacodyn* 1960, 129, 273–289

Price Evans, D A Genetic variations in the acetylation of isoniazid and other drugs *Ann N Y Acad Sci* 1968, 151, 723–733

Schildkraut J J & S S Kety Biogenic amines and emotion *Science* 1967, 156, 21–30

Siwers, B, D Tuck, B Hamberger, U Freyschuss, T Malmfors & F Sjoqvist A quantitative approach to the initial clinical trial of tricyclic anti depressants comparison of Leo 640 and nortriptyline *Eur J Clin Pharmacol* 1971, 3, 12–17

Sjoqvist, F Lakemedelsbiverkningar mekanismer bakom dosberoende biverkningar *Svensk Lakartidn* 1968, 65, 34–44

Sjoqvist, F, P G Bergfors O Borgå, M Lind & H Ygge Plasma disappearance of nortriptyline in a newborn following placental transfer from an intoxicated mother – evidence for drug metabolism *J Pediatrics* 1971, in press

Sjoqvist F, F Berglund, O Borgå, W Hammer, S Andersson & C Thorstrand The pH dependent excretion of monomethylated tricyclic antidepressants in the dog and in man *Clin Pharmacol Therap* 1969a, 10, 826–833

Sjoqvist, F, W Hammer, O Borgå & D L Azarnoff Pharmacological significance of the plasma level of monomethylated tricyclic antidepressants In *Excerpta Med Internat Congr Ser* 1969b, 180, 128–136 Proc Coll Internat Neuropsychopharmacol, Tarragona, 1968

Sjoqvist, F, W Hammer, C M Idestrom, M Lind, D Tuck & M Åsberg Plasma level of monomethylated tricyclic antidepressants and side effects in man In *Excerpta Medica Internat Congr Ser* 1968, 145, 246–257

Smith, T W, V P Butler & E Haber Determination of therapeutic and toxic serum digoxin concentrations by radioimmunoassay *New Engl J Med* 1969, 281, 1212–1216

Wagner, J G, J I Northman, C D Alway & O S Carpenter Blood levels of drug at the equilibrium state after multiple dosing *Nature* 1965, 207, 1301–1302

Åsberg M, B Cronholm, F Sjoqvist & D Tuck The correlation of subjective side effects with plasma concentrations of nortriptyline *Brit Med J* 1970 4, 18–21

Åsberg M, B Cronholm, F Sjoqvist & D Tuck Relationship between plasma level and therapeutic effect *Brit Med J* 1971a, in press

Åsberg M, D A Price Evans & F Sjoqvist Genetic control of nortriptyline kinetics in man – A study of the relatives of propositi with high plasma concentrations *J Med Gen* 1971b, 8 129–135

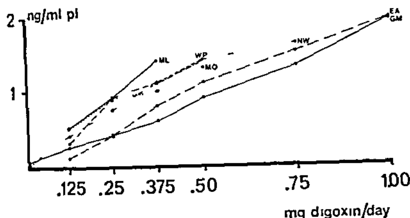


Fig. 3 Steady state plasma digoxin concentration in six patients which during 14 days periods have been treated with successively increasing doses of digoxin

into erythrocytes in the presence of known amounts of digoxin is illustrated in fig 2 A standard curve can from these values be constructed and the inhibition of ^{86}Rb uptake brought about by the patient samples can then be plotted against this curve, allowing a calculation of their glycoside content.

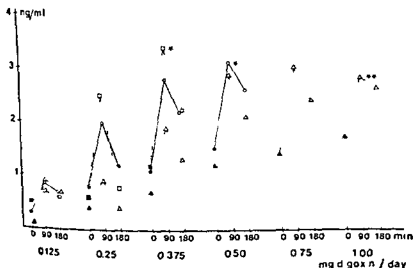


Fig. 4. Plasma digoxin concentration in three patients before and 90 and 180 minutes after administration of the actual maintenance dose of the period.
* = toxic symptoms
** = because of toxic symptoms only 0.5 mg was given

Fig 1 The ^{86}Rb method for estimation of digitalis glycosides in plasma

1 2 ml of plasma are extracted with 5 ml of dichloromethane
2 The extract is evaporated to complete dryness and redissolved in NaCl glucose solution (1 ml)
3 0.5 ml of washed, packed erythrocytes are added
4 Pre incubation at 37° for 2 hours
5 Addition of 5 µci ^{86}Rb
6 Main incubation at 37° for 1 hour
7 Centrifugation and washing of the erythrocytes
8 γ activity (1 08 MeV) in erythrocytes is registered

sensitivity and was insufficient regarding both accuracy and precision After a systematic study many hidden errors have been unveiled, and based upon the results obtained during this study, modifications of the method have been introduced, which highly increased both its sensitivity, accuracy, and precision (BERTLER & REDFORS 1970) The lower limit of sensitivity seems now to be 0 2 (2 SD) ng digoxin/ml of plasma, thus allowing estimation of the very low plasma concentrations wich are obtained after therapeutic oral doses of the glycoside The recovery is 98 0 % ± 9 8 (mean ± S D) With the method various types of cardiac glycosides can be determined The procedure is shortly presented in fig 1 The decreased transport of ^{86}Rb

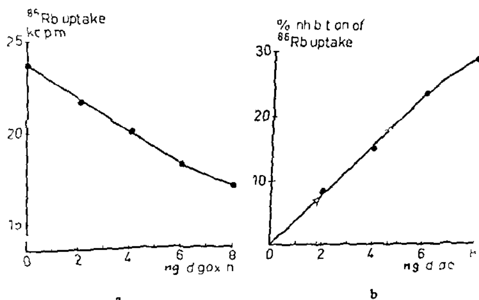


Fig 2a Decrease in ^{86}Rb uptake of erythrocytes at increasing concentration of digoxin

Fig. 2b The constructed standard curve Two patient samples are plotted against the curve in order to calculate their glycoside content

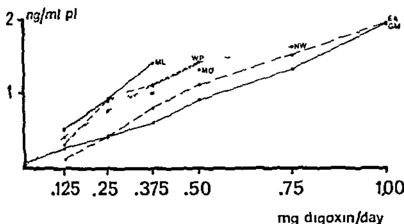


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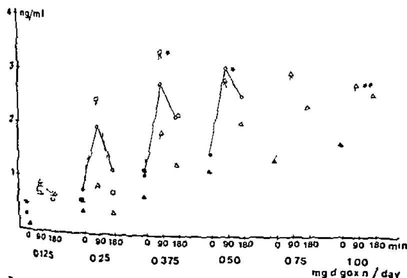


Fig. 4. Plasma digoxin concentration in three patients before and 90 and 180 minutes after administration of the actual maintenance dose of the period.
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In a clinical dose-response study, performed single blind, patients with atrial fibrillation were during a series of 14 days periods treated with successively increasing doses of digoxin, from 0 125 mg/day up to doses which gave toxic symptoms All patients had normal renal function as judged from creatinine clearance tests At the end of each period, the patients were clinically examined and the digoxin concentration in plasma was estimated in samples drawn 24 hours after the last dose administered (steady state concentration") In this way 14 patients have been examined, but because of the methodological problems at the beginning of the investigation, complete series of digoxin analysis can be given for only the last six patients The results obtained are given in fig. 3, from which it is evident that the basic concentration slowly increases with increasing doses of digoxin Mean values

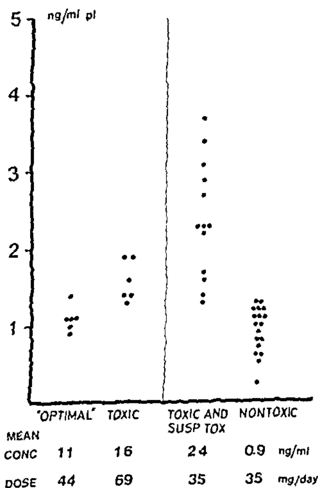


Fig 5 To the left plasma digoxin concentrations in a patient group treated with increasing doses of digoxin up to toxic symptoms appeared
To the right plasma digoxin concentrations in patients with toxic symptoms compared with nontoxic digitalized patients

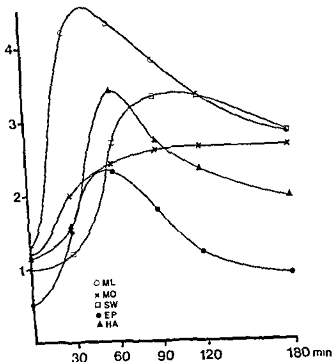


Fig. 6 Plasma digoxin concentration in five patients treated with 0.375 mg digoxin/day for 14 day or more Samples were drawn before and 30, 60, 90, 120 and 180 minutes after administration of the daily dose

of 0 7 (0 4–0 9) and 1 2 (0 9–1 4 ng/ml of plasma are registered at doses of 0 25 and 0 50 mg digoxin/day These results are in good aggreement with those obtained with the radioimmunoassay (SMITH *et al* 1969)

The interindividual differences in digitalis sensitivity is well known in the clinic In fig 4 the plasma digoxin concentrations in three patients with varying reactivity to the glycoside are given As can be seen, a patient which did tolerate big doses of digoxin increased his plasma levels more slowly than a patient more sensitive to the drug. This difference was accentuated 90 and 180 minutes after administration of the actual maintenance dose Toxic symptoms appeared at doses between 0 375 up to 1 0 mg/day Despite these dose difference the plasma digoxin levels were rather well gathered with a mean of 1.5 ng ml (fig 5) In this patient group, where every individual had been treated in a strictly uniform way, there was a good separation between the values obtained at "optimal" and toxic digoxin doses The predominant toxic symptoms were extracardiac, such as nausea, vomiting etc.

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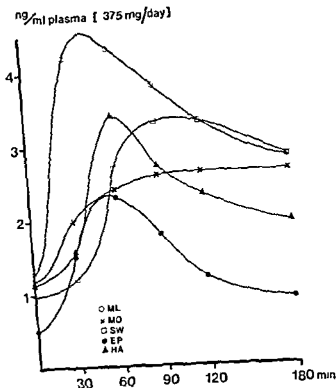


Fig 6 Plasma d goxin concentration in five patients treated with 0.375 mg digoxin/day for 14 day or more Samples were drawn before and 30, 60, 90, 120 and 180 minutes after administration of the daily dose.

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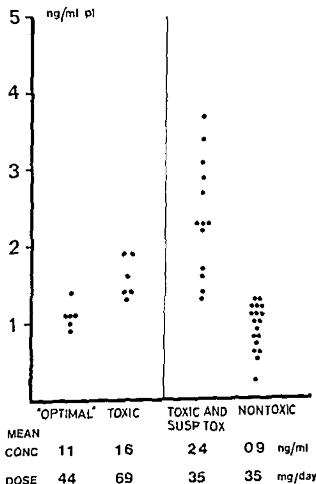


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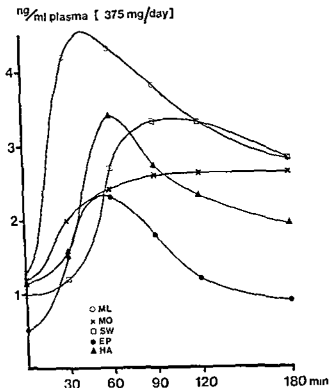


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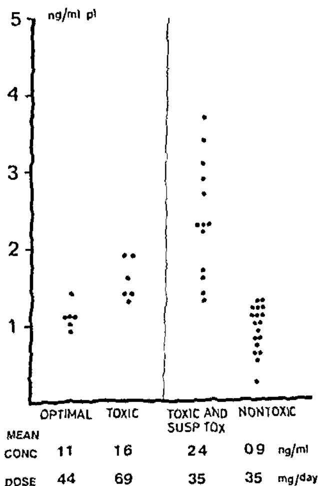


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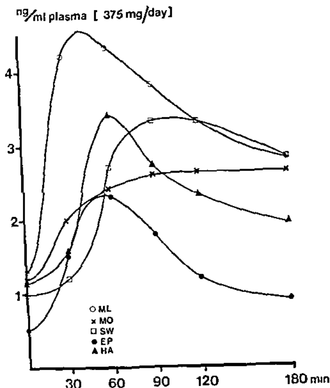


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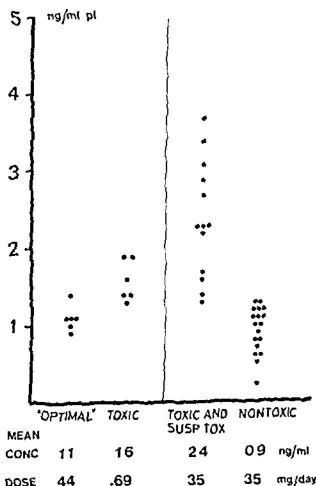


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Marcus, F I, L Burkhalter, C Cuccia, J Pavlovich & G G Kapadia Administration of tritiated digoxin with and without a loading dose – A metabolic study *Circulation* 1966 **34**, 865–874

Schatzmann, H J Hertzglykoside als Hemmstoffe fur den aktiven Kalium und Natriumtransport durch die Erythrocytenmembran *Helv Physiol Acta* 1953, **11**, 346–354

Smith T W, V P Butler & E. Haber Determination of serum digoxin concentrations by radioimmunoassay *New Engl J Med* 1969, 281, 1212–1216

Wilson, W S Newer concepts regarding digitalis therapy *Med Clin North Amer* 1969 **53** 1279–1286

When examining a more heterogenous patient material with clear or suspect intoxication, as for example from a medical intense care unit, higher and more scattered digoxin values can be found Other factors, such as hypopotassemia, varying status of the myocardium etc may here contribute to differences in the sensitivity to the drug There is, however, no overlapping between these values and those obtained from digitalized but non toxic outpatients (fig 5)

When determining the plasma concentration of digoxin, and probably also the other cardiac glycosides, it is of course very important to define the interval between the last dose administered and the time for plasma sampling In many cases failure to do this had made it difficult to evaluate results presented in literature More detailed studies about the changes in plasma digoxin content after administration of the daily maintenance dose to digitalized patients have revealed great interindividual differences (fig 6) In this figure the glycoside values before and up to three hours after oral administration of 0 375 mg of digoxin are given All patients were investigated at the same time in the morning and about 1½ hour after a standardized light meal It is possible that the curves presented only in part reflect the absorption of the glycoside in these patients, but nevertheless they may offer a new possibility to attack the problem about the interindividual variations in sensitivity to cardiac glycosides

REFERENCES

Bertler, A. & A Redfors An improved method of estimating digoxin in human plasma *Clin Pharmacol Therap* 1970 11, 665

Binnion, P F, L M Morgan, H M Stevenson & E Fletcher Plasma and myocardial digoxin concentrations in patients on oral therapy *Brit Heart J* 1969 31, 636–640

Binnion, P F, L M Morgan & E Fletcher New ways with digoxin (Letter to the editor) *Lancet* 1970, I, 678

Coiner, D C C Bangs, J R Walsh & L W Rilzmann Serum cardiac glycoside assay method and possible clinical use *J Nucl Med* 1968 9 377

Doherty, J E The clinical pharmacology of digitalis glycosides A review *Amer J Med Sci* 1968 255, 382–414

Evered, D C. New ways with digoxin (Letter to the editor) *Lancet* 1970 I 623

Grahame Smith, D G & M S Everest Measurement of digoxin in plasma and its use in diagnosis of digoxin intoxication *Brit Med J* 1969 I 286–289

Love, W D & G E Burch A comparison of potassium[42], rubidium[86] and cesium[134] as tracers of potassium in the study of cation metabolism of human erythrocytes *in vitro J Lab Clin Med* 1953, 41, 351–362

Lowenstein, J M & E. M Corrill An improved method for measuring plasma and tissue concentrations of digitalis glycosides *J Lab Clin Med* 1966 67, 1048–1052

Lyon A F & A C. De Graff Reappraisal of digitalis Part V Evaluation of criteria for determining effect of digitalis in man *Amer Heart J* 1967, 73 134–136

factors modifying availability for absorption are the most frequent sources of generic inequivalence. Not only must drugs be equally available, but the absorption characteristics must be the same. While one dosage form could be absorbed slowly so that effective tissue concentrations of drugs are never achieved, very rapid absorption of another preparation could cause exaggerated and transient effects with an increased risk of toxicity

Shape, size, colour, taste, solubility at different pH values, the effects of other formulation ingredients, binders, lubricants, granulation, particle size, stability, age, tablet compression and thickness, and the type of coating may all influence therapeutic equivalence, and many of these factors have already been discussed at length by previous speakers. The appearance of a preparation may seem irrelevant, but the placebo effect is often more important in clinical practice than we care to admit. The response of patients to a given drug may even vary according to the colour of the tablets (SCHAPIRA *et al.* 1970)

Physico-chemical factors influencing drug availability have been extensively studied *in vitro*, using highly sophisticated techniques. On the other hand, clinical investigation of drug equivalence has been grossly neglected. This is an extraordinary state of affairs, although it may well turn out that ignorance is bliss. Measurement of therapeutic equivalence of drugs in clinical medicine is difficult because of biological variation in the effects of drugs, the natural course of the underlying diseases, and the frequent lack of objectivity in the measurement of drug effects. In practice, the availability for absorption is usually assessed by estimation of drug concentrations in plasma and urine, and if comparable levels are obtained with different formulations, it is assumed that they are therapeutically equivalent. However there are pitfalls in this approach because drug activity is not necessarily related to drug concentrations in plasma. In addition the plasma concentration of a drug depends not only on absorption, but also on individual characteristics and the kinetics of drug distribution, metabolism and excretion. The following review is far from complete but the examples given illustrate several mechanisms by which generic inequivalence can occur

Clinical and experimental studies

Tolbutamide – tablet disintegration. Five volunteers were given 2 tablets (1 0 g) of a branded preparation of tolbutamide, and another 5 received 2 tablets of the same formulation containing only half the amount of disintegrating agent (Vee Gum) The *in vitro* disintegration time was prolonged from 2 to 7 6 min., and the dissolution "rate" from 3 8 to 103 min. Both formulations met the U.S P specifications completely There were

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Generic Inequivalence – Clinical Observations

By

Laurie F. Prescott and John Nimmo

Abstract Different preparations of a drug containing the same amount of active chemical in stable form are "chemically equivalent". Unless they have identical biological activity under clinical conditions, however, they cannot be considered generic equivalents Differences in drug formulation affecting availability for absorption are a frequent cause of generic inequivalence The truth of the matter is that although drug formulation has been studied extensively *in vitro*, the clinical significance and extent of generic inequivalence is unknown Minor changes in the formulation of prednisone, diphenylhydantoin and dicoumarol produced alarming variation in clinical effect, although each preparation conformed to official standards In experimental studies, generic inequivalence has been demonstrated in preparations of tolbutamide, phenacetin, nitrofurantoin, chloramphenicol, tetracycline and a combination of triamterene and hydrochlorothiazide In some cases, inequivalence could be explained by differences in tablet disintegration, dissolution rate and particle size Preliminary studies with different dosage forms of paracetamol suggest that significant generic inequivalence might occur with some drugs only in certain individuals ("slow absorbers") The risk of generic inequivalence is increased with enteric coated and delayed release preparations since absorption may be poor and erratic Apart from active drug, modern tablets and capsules usually contain many other substances Some of these may occasionally contribute to generic inequivalence in the form of unsuspected toxicity

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It is widely assumed that non-proprietary generic preparations are as effective as branded drugs simply because they are chemically equivalent, and there is pressure on physicians to prescribe the cheaper generic drugs, particularly for welfare or National Health Service patients In recent years several instances of drug formulation failure have been reported some of these involving manufacturers of the highest international reputation

"Chemical equivalence" means that preparations merely contain the same amounts of active chemical in a stable form in accordance with official standards But drugs can only be considered "generic" equivalents if they have absolutely identical biological activity under clinical conditions With oral dosage forms this depends largely on the kinetics of absorption, and

Table I.

Mean serum tetracycline concentrations (µg/ml) in groups of volunteers receiving 11 different commercial preparations of tetracycline (fluorometric assay) (After ALTMANN *et al.* 1968).

Preparation	after 2 hrs	after 4 hrs
A Capsules with metaphosphate	1.81	1.93
B Tablets	1.54	1.52
C Capsules	0.95	1.26
D Capsules	1.85	2.31
E Capsules	1.09	1.47
F Tablets with bitartrate	1.85	2.00
G Capsules	1.88	2.02
H Coated tablets	0.04	0.33
I Capsules	1.10	1.09
K Tablets	0.84	0.81
L Tablets with bitartrate	1.72	1.91

latter, mean values ranged from 0.04 to 1.88 µg/ml at 2 hrs – a 47 fold difference (table 1). On occasion, manufacturers have had to withdraw stocks of tetracycline because of failure of absorption. 40 million oxytetracycline capsules made by 9 drug companies were recently recalled from the American market by the FDA because many batches failed to produce satisfactory blood levels (ANNOTATION 1969).

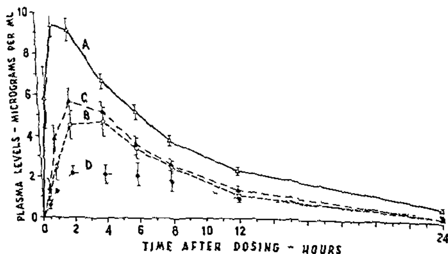


Fig. 2. Mean plasma levels for groups of 10 human subjects receiving 0.5 g oral doses of chloramphenicol. Preparations A, B, C, or D (colorimetric assay). Vertical bars represent one standard error on either side of the mean. (GLAZKO *et al.* 1968)

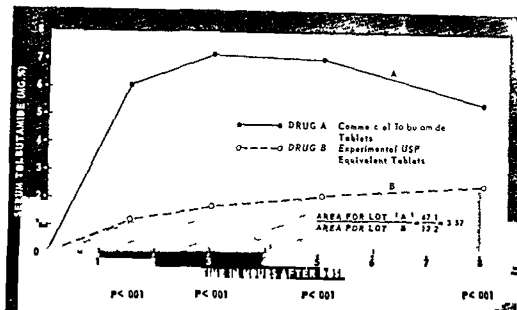


Fig 1 Serum tolbutamide levels after administration of commercial tolbutamide (orinase) and experimental drug Results were achieved in a carefully controlled two way crossover study in 10 normal adult volunteers (Varley 1968)

marked differences in the serum tolbutamide concentrations with the 2 formulations The area of the blood level-time curves with the branded tablet was 3 75 times that for the tablet with reduced amounts of disintegrating agent (fig 1) This difference was reflected in the respective mean blood sugar concentrations (Varley 1968)

Prednisone – a case report Campagna *et al* (1963) reported the case of a 25 year old woman with Familial Mediterranean Fever whose episodic symptoms invariably responded to 20 mg prednisone daily for 2–3 days On one occasion she was given a different brand of prednisone, and after treatment for 3 days there was no clinical response She was then transferred back to her usual brand of prednisone, with almost complete resolution of her symptoms in 24 hrs The suspect tablets were found to meet U S P requirements in respect of drug content and tablet disintegration, but dissolved much more slowly than the effective tablets The latter released 50 per cent of their content 20 times as rapidly as the ineffective tablets

Tetracycline Tetracycline absorption is modified by divalent metal ions and by complex formation with citrate, tartrate and phosphate Changes in the formulation of tetracyclines can therefore result in marked changes in availability, and many examples have been recorded Altmann *et al* (1968), for instance studied the absorption of 11 different commercial tetracycline preparations in volunteers who were stated to be "in fairly good health" Following single 500 mg doses, serum tetracycline concentrations were measured by microbiological and fluorometric methods With the

Table 2

Mean maximum plasma concentrations of phenacetin and free paracetamol following oral administration of different preparations of phenacetin in 6 healthy volunteers.

Phenacetin preparation	Mean maximum plasma phenacetin concentration, μg/ml (± S.E.M.)	Mean maximum plasma free paracetamol concentration, μg/ml (± S.E.M.)	Dose (g)
Fine + Tween 80	13.5 ± 3.4	13.7 ± 1.8	1.5
Fine	9.6 ± 3.3	12.7 ± 1.2	1.5
Medium	3.3 ± 2.1	10.6 ± 1.8	1.5
Coarse	1.4 ± 0.6	10.0 ± 1.5	1.5
Tablet "A"	2.7 ± 0.8	7.8 ± 1.3	0.65
Tablet "B"	2.5 ± 1.0	8.7 ± 0.6	1.0

sules in normal volunteers. Following a dose of 500 mg the mean maximum plasma concentrations for the 4 preparations were 10.9, 5.2, 6.3 and 2.7 μg/ml, (fig. 2) and the corresponding 48 hr urinary recoveries of the drug were 85, 60, 70 and 33 per cent. The better absorbed preparations were found to have more rapid dissolution rates. It was subsequently established that variations in particle size were not responsible for the differences in absorption of these preparations (AGUIAR *et al.* 1968).

Particle size is likely to be an important factor in drug absorption, particularly in the case of compounds with limited aqueous solubility. This subject has recently been reviewed by FINCHER (1968). The absorption of griseofulvin (ATKINSON *et al.* 1962) and spironolactone (VENNING 1964), for instance, depends on particle size. CONKLIN & HAILEY (1969) showed that microcrystals of nitrofurantoin in tablets were better absorbed than macrocrystals in capsules. They attributed this to the differences in particle sizes but apparently overlooked the possibility of differences due to formulation as tablets or capsules.

Phenacetin. We have studied the effects of particle size on the absorption of phenacetin (PRESCOTT *et al.* 1970). In separate experiments 6 volunteers each received 1.5 g of phenacetin U.S.P. as a fine suspension (particle size less than 75 μ) with or without Tween 80, a medium suspension (150–180 μ) and a coarse suspension (greater than 250 μ). They also received 4 tablets of 2 proprietary combination analgesics containing phenacetin. The highest plasma concentrations of phenacetin were observed after the fine suspensions followed by the medium and coarse in decreasing order (fig. 3). There were corresponding but less marked differences in the plasma concentrations of paracetamol (the major metabolite

Diphenylhydantoin (phenytoinum NFN) Minor changes in formulation may cause disproportionate effects on availability Two years ago there was an outbreak of cases of phenytoin toxicity in Australia following a change in the manufacture of the 100 mg capsules (lactose was substituted for calcium sulphate as the excipient) In some patients the manifestations of phenytoin intoxication were such that posterior fossa tumours were suspected (EADIE *et al* 1968, LANDY 1968) It was never finally established that the formulation changes were responsible for the toxicity, but the circumstances were most suspicious

Dicoumarol A change in the size of dicoumarol tablets was followed by reports of reduced therapeutic effects The manufacturers responded by introducing a new formulation in which the drug was very finely divided This resulted in numerous complaints of excessive anticoagulant activity, and haemorrhage occurred in some patients (LOZINSKI 1960) Generic inequivalence in this group of drugs could be disastrous

Chloramphenicol GLAZKO *et al* (1968) reported important differences in the absorption of 4 commercial preparations of chloramphenicol cap

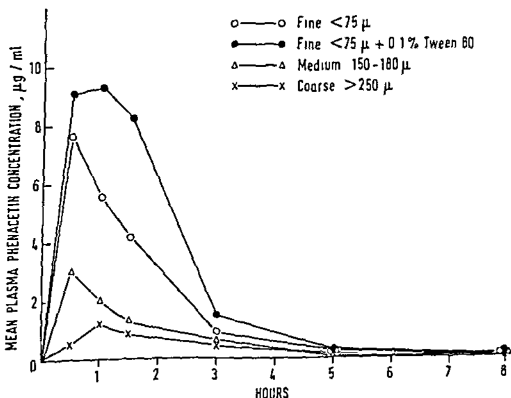


Fig 3 Mean plasma phenacetin concentrations in 6 adult volunteers following administration of 1.5 g doses of phenacetin in suspension of different particle sizes (PRESCOTT *et al* 1970)

fervescent preparation (Paragesic® (Sandoz), containing 500 mg paracetamol 20 mg pseudoephedrine and 10 mg caffeine in each effervescent tablet) or as 3 plain tablets marketed by a well known pharmaceutical company The mean peak plasma concentrations of free paracetamol occurred at 30 min with the effervescent preparation and the suspension, but at 90 min with the tablets. There was a corresponding delay in the urinary excretion of free and conjugated paracetamol after the tablets.

Although our studies are incomplete, we have found that normal individuals can be divided into consistently "rapid" and "slow" absorbers of the drug. All three preparations gave virtually identical blood level curves in the "rapid" absorbers although the peak concentration was delayed a further 30 min with the tablets (fig. 4) In contrast, the differences between the preparations were marked in the "slow" absorbers (fig. 5) The proportion of the dose excreted in the urine within 4 hrs was 25 per cent with the tablet, 30 per cent with the suspension and 35 per cent with the effervescent preparation Corresponding figures for the period 4–12 hrs after administration were 39 per cent, 30 per cent and 30 per cent. The overall recovery of the drug in the urine was similar with all the preparations and the so-called "poor" absorbers of paracetamol described by GWILT *et al* (1963) seem rather to be "slow" absorbers. Furthermore "slow" absorption is relative and is a significant problem

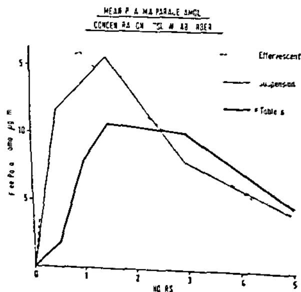


Fig. 5 Mean plasma concentrations of free paracetamol in "slow" absorbers receiving 1.5 g of parcetamol in the same preparations as in fig. 4

Table 3

The relationship between plasma phenacetin concentrations and central nervous system toxicity in 6 normal volunteers receiving 6 different formulations of phenacetin

CNS effects	Mean maximum plasma phenacetin concentration (μg/ml ± S E M)	N
Marked	10 6 ± 2 2	11
Mild	8 0 ± 3 9	5
None	2 0 ± 0 4	20

of phenacetin), and in the urinary recovery of paracetamol (table 2) There were also differences in the rate of absorption of phenacetin with the tablets In this study we encountered a high incidence of C N S effects such as lightheadedness, dizziness, weakness and a feeling of detachment *from the surroundings These effects came on very rapidly, usually oc-*curred after administration of the fine suspensions of the drug and were associated with high plasma concentrations of phenacetin (table 3) This is a good example of experimental inequivalence giving rise to drug toxicity

Paracetamol We have also looked at the absorption of different dosage forms of paracetamol Healthy volunteers were given 1 5 g of paracetamol either as a suspension (200 mg/ml with 0 2 per cent tragacanth), an ef-

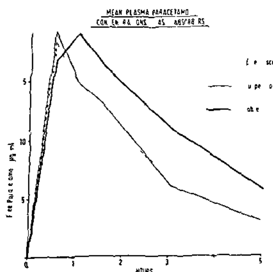


Fig 4 Mean plasma free paracetamol concentrations in 'rapid" absorbers receiving 1.5 g of paracetamol as a suspension, an effervescent preparation or plain tablets

some enteric coatings have been shown to be hopelessly unpredictable, even though they met official specifications In some cases, enteric coated drugs were not absorbed at all, (fig. 7) and in others, the absorption was poor and erratic (LEVY & HOLLISTER 1964b, LEONARDS & LEVY 1965, CLARKE & LASAGNA 1965, SVEDMYR & HARTHON 1970) For example, LEVY & JUSKO (1967) found that absorption of one brand of enteric coated aspirin tablets occurred 7–19.5 hrs after administration, and was so slow as to be therapeutically ineffective After 24 hrs, only 2–64 per cent (mean 28 per cent) of the administered dose was recovered in the urine

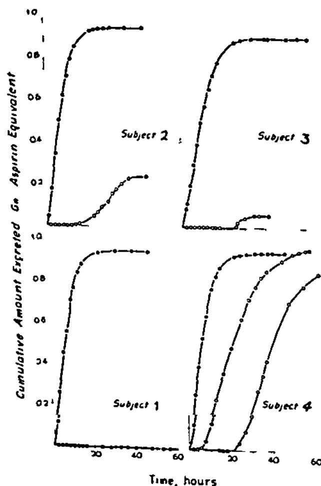


Fig. 7 Cumulative excretion of salicylate expressed in terms of aspirin after ingestion of 1 g aspirin in solution (●) and in enteric coated tablets (O) (LEVY & HOLLISTER 1964)

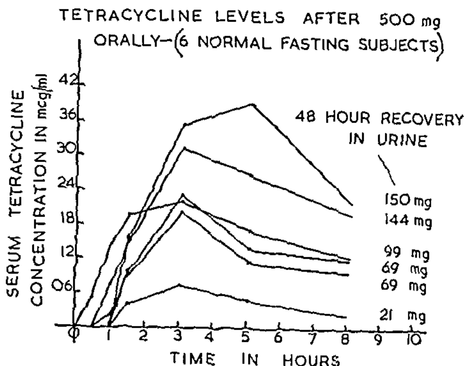


Fig 6 Serum tetracycline concentrations (microbiological assay) and 48 hr urinary tetracycline recovery following administration of 500 mg tetracycline in coated tablets in 6 fasting healthy volunteers

only with certain dosage forms of the drug This raises the interesting possibility that generic inequivalence might occur with some drugs only in certain individuals

Levy & Hollister (1964a) have described "slow and "rapid" absorbers of aspirin, and we have observed a similar phenomenon with absorption of phenacetin With Dr J A Raeburn, we have recently encountered striking individual variation in the absorption of an expensive brand of coated tablets of tetracycline in healthy volunteers under carefully controlled test conditions (fig 6) The clinical implications of such extreme individual variation are obvious, and are at least as important as differences in drug metabolism In a given case, such variation in absorption may be much more important than generic inequivalence

Compounding the errors

Enteric coated and delayed release preparations Although most of us here would not accept an enteric coated or delayed release formulation as generically equivalent to a conventional dosage form, physicians do not generally make any distinction Apart from the different absorption kinetics

coated and delayed release formulations, and in general they are best avoided

Drug combinations It cannot be guaranteed that drugs given as combination preparations will be equivalent to the same drugs given separately There are many possible interactions (PRESCOTT 1969) For example, p-aminosalicylic acid (PAS) not only inhibits the metabolism of isoniazid, but may also interfere with its absorption The 2 drugs are often formulated together, and some preparations of PAS have greater effects than others The overall effects on the plasma concentrations of isoniazid may be complex (TIITINEN & MATTILA 1968)

Capsules – an answer to the pharmaceutical maiden's prayer? Since tablet manufacture involves so many important variables, it is sometimes assumed that capsules are a more reliable dosage form This may be so in some cases, but there are exceptions TANNENBAUM *et al* (1968) compared the absorption of a combination of triamterene and hydrochlorothiazide in a 2 1 ratio formulated as tablets or capsules in 24 volunteers Only 12–18 per cent of the triamterene was recovered in the urine in 24 hrs with the capsule, compared with 38–56 per cent with the tablet The corresponding figures for hydrochlorothiazide were 27–31 per cent and 52–59 per cent respectively (fig 8) The peak urinary excretion of electrolytes was constantly and significantly higher with the tablets than with the capsules

Despite the widespread use of capsule formulations, relatively little attention seems to have been given to their properties. In particular, the *in vitro* dissolution of drugs from capsules is markedly influenced by the nature of fillers and lubricants (SAMYN & JUNG 1970) In this context it is important to consider generic inequivalence in clinical trials, especially trials of new drugs It is common practice to make up "special" batches of drugs for clinical trials, and in the case of new drugs it is convenient to use capsules until the final formulation of the drug has been decided by the manufacturer If these "special" batches of drugs for clinical trials are not identical in their characteristics to the final formulation (and this includes any established drugs used for therapeutic comparisons), then the results could be quite misleading (FREESTONE 1969)

Toxic impurities Formulation constituents other than active drug may contribute to clinically significant generic inequivalence in the form of unsuspected toxicity FREESTONE (1969) has drawn attention to the amazing selection of different substances which may find their way into the modern tablet (table 4) These may occasionally give rise to trouble For example, renal tubular acidosis was attributed to toxic degradation products of improperly stored outdated tetracycline It was suggested that degradation was accelerated by the presence of citric acid in the capsules, and stability was improved by the replacement of citric acid by lactose (FULOP &

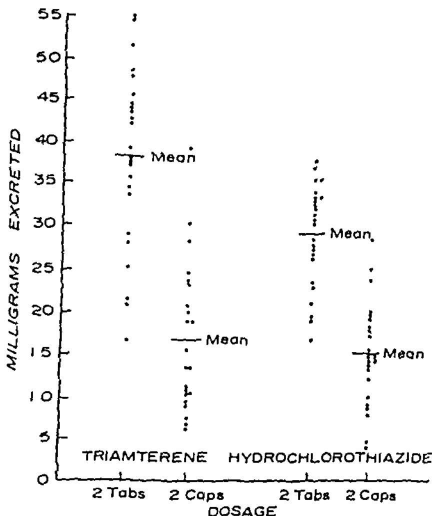


Fig 8 Each dot shows the amount of drug excreted in the urine by a volunteer subjects in the 24 hr period following ingestion of the indicated dosage form (TANNENBAUM *et al* 1968)

ORZACK *et al.* (1969) found differences in objective psychomotor test scores in volunteers and in schizophrenics given 300 mg of chlorpromazine either as a single dose in a sustained release capsule or in repeated doses, and HOLLISTER *et al* (1970) observed erratic and poor absorption of chlorpromazine from long acting capsules. Although desirable in principle with a few drugs, the use of enteric coatings and sustained release formulations seems to be getting out of hand. In the U.K for instance, it is possible to prescribe sustained release formulations of phenobarbital (phenemalum NFN), and not long ago enteric coated *thyroid*, of all drugs, was available in the U.S.A. Use of the latter preparation was associated with clinical deterioration, and intact enteric coated thyroid tablets were passed in the stools (CLARKE & LASAGNA 1965). Physicians should be wary of enteric

and very little, if anything, is known of the equivalence of drugs given as syrups, aerosols, ointments, and as preparations for injection or topical application We may have appeared to judge the pharmaceutical industry too harshly, but it is clear that complex *in vitro* studies of drug formulations cannot be relied upon to predict the performance of drugs in clinical practice Furthermore, the existing official standards may do more harm than good by engendering a false sense of security There is an appalling lack of information on the equivalence of drugs in the very situation where it is most needed – in patients with disease

Summary

Different preparations of a drug containing the same amount of active chemical in stable form are "chemically equivalent" Unless they have identical biological activity under clinical conditions, however, they cannot be considered generic equivalents Differences in drug formulation affecting availability for absorption are a frequent cause of generic inequivalence

The truth of the matter is that although drug formulation has been studied extensively *in vitro*, the clinical significance and extent of generic inequivalence is unknown Minor changes in the formulation of prednisone, diphenylhydantoin and dicoumarol produced alarming variation in clinical effect, although each preparation conformed to official standards

In experimental studies, generic inequivalence has been demonstrated in preparations of tolbutamide, phenacetin, nitrofurantoin, chloramphenicol, tetracycline and a combination of triamterene and hydrochlorothiazide In some cases inequivalence could be explained by differences in tablet disintegration, dissolution rate and particle size Preliminary studies with different dosage forms of paracetamol suggest that significant generic inequivalence might occur with some drugs only in certain individuals ("slow absorbers")

The risk of generic inequivalence is increased with enteric coated and delayed release preparations since absorption may be poor and erratic Apart from active drug, modern tablets and capsules usually contain many other substances Some of these may occasionally contribute to generic inequivalence in the form of unsuspected toxicity

REFERENCES

Aguiar A. J., L. M Wheeler, S Fusari & J E. Zelmer Evaluation of physical and pharmaceutical factors involved in drug release and availability from chloramphenicol capsules, *J pharm Sci* 1968, 57, 1844–1850

Table 4.

Inactive substances which might be found in a hypothetical sugar coated tablet (after FREESTONE 1969)

Constituent	Content (g)
Tablet core	
ABC 789 (active substance)	0 0050
Dimethylsilicone oil	0 00050
Polyethylene glycol 6000	0 00050
Benzenesulphonic acid	0 00205
Polyvinylpyrrolidone	0 0030
Sucrose	0 0030
Talc	0 0030
Maize starch	0 0060
Lactose	0 026950
Coating mass	
Indigotine pigment	0 000171
Cetyl alcohol	0 000017
Arachis oil hydrogenated	0 000017
Stearic acid	0 000104
Polyethylene glycol 6000	0 000104
Silicic acid	0 000174
Polyvinylpyrrolidone	0 000174
Cellulose	0 000304
Titanium dioxide	0 000869
Talc	0 001035
Sucrose	0 007031
Total tablet weight	0 060

DRAPKIN 1965) Some brands of penicillin may contain more allergenic residues than others (STEWART 1967) and recently, CAMERON *et al* (1970) have reported that the majority of barbiturates available in the U K are contaminated with barbital (diemalum NFN) Whilst this was usually of no clinical significance, accumulation of barbital occurred in patients with renal failure Plasma barbital concentrations of up to 13 2 μg/ml were found in patients with renal failure receiving pentobarbital and no other barbiturate

Conclusions

The incidence and ultimate clinical significance of generic inequivalence is unknown We have concentrated on a few drugs given by the oral route

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Maize starch	0 0060
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Coating mass	
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Cetyl alcohol	0 000017
Arachis oil hydrogenated	0 000017
Stearic acid	0 000104
Polyethylene glycol 6000	0 000104
Silicic acid	0 000174
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Prescott, L F, R. F Steel & W R. Ferrier The effects of particle size on the absorption of phenacetin in man A correlation between plasma concentration of phenacetin and effects on the Central Nervous System *Clin Pharmacol Therap* 1970, **11**, 496–504

Samyn, J C. & W Y Jung *In vitro* dissolution from several experimental capsule formulations. *J pharm Sci* 1970, 59 169–175

Schapira, K., H. A. McClelland, N R. Griffiths & J Newell Study on the effects of tablet colour in the treatment of anxiety states. *Brit med J* 1970, **2**, 446–449

Steward, G T Allergenic residues in penicillin. *Lancet* 1967, **1**, 1177–1183

Svedmyr, N & L. Harthon Comparison between the absorption of nicotinic acid and pentaerythritol tetranicotinate (Perycit) from ordinary and enterocoated tablets *Acta pharmacol et toxicol* 1970, **28**, 66–74

Tannenbaum, P J., E. Rosen, T Flanagan & A P Crosley The influence of dosage form on the activity of a diuretic agent *Clin Pharmacol Therap* 1968, **9**, 598–604

Tutinen H & M J Mattila Modification of the serum isoniazid levels by different preparations of para aminosalicylic acid *Arzneimittel Forsch* 1968 **18**, 623–625

Varley, A B The generic inequivalence of drugs *J Amer med Ass* 1968, **206**, 1745–1748

Venning, G R. Absorption of steroids with special reference to spironolactone – In *Absorption and distribution of drugs* Ed. T B Binns Williams & Wilkins Co Baltimore 1964, p 150–156

Altmann, A E, H Beeuwkes, P J Brombacher, H J Buytendijk, A H J Gijzen & F P V Maesen Serum levels of tetracycline after different administration forms *Clin chim Acta* 1968, **20**, 185–188

Annotation 40 million antibiotic capsules recalled *J Amer med Ass* 1969, **210**, 2350

Atkinson, R M, C Bedford, K J Child & E G Tomich Effect of particle size on blood griseofulvin levels in man *Nature* (Lond) 1962, **193**, 588–589

Cameron, J S, P A Toseland, J F Read, M Bewick, C S Ogg & F G Ellis Accumulation of barbitone in patients on regular haemodialysis *Lancet* 1970, **1**, 912–914

Campagna, F A, G Cureton, R A Mirigian & E Nelson Inactive prednisone tablets U S P XVI *J pharm Sci* 1963, **52**, 605–606

Clarke, R L & L Lasagna How reliable are enteric coated aspirin preparations? *Clin Pharmacol Therap* 1965, **6**, 568–574

Conklin, J D & F J Hailey Urinary drug excretion in man during oral dosage of different nitrofurantoin formulations *Clin Pharmacol Therap* 1969, **10**, 534–539

Eadie, M J, J M Sutherland & J H Tyrer Dilantin overdosage *Med J Aust* 1968, **2**, 515

Fincher, J H Particle size of drugs and its relationship to absorption and activity *J pharm Sci* 1968, **57**, 1825–1835

Freestone, D S Formulation and therapeutic efficacy of drugs used in clinical trials *Lancet* 1969, **2**, 98–99

Fulop, M & A Drapkin Potassium depletion syndrome secondary to nephropathy apparently caused by 'outdated tetracycline' *New Engl J Med* 1965, **272**, 986–989

Glazko, A J, A W Kinkel, W C Alegnani & E L Holmes An evaluation of the absorption characteristics of different chloramphenicol preparations in normal human subjects *Clin Pharmacol Therap* 1968, **9**, 472–483

Gwilt, J R, A Robertson, L Goldman & A W Blanchard The absorption characteristics of paracetamol tablets in man *J Pharm Pharmacol* 1963, **15** 445–453

Hollister, L E, S H Curry, J E Derr & S L Kanter Studies of delayed action medication V Plasma levels and urinary excretion of four different dosage forms of chlorpromazine *Clin Pharmacol Therap* 1970 **11** 49–59

Landy, P J Dilantin overdosage *Med J Aust* 1968, **2**, 639

Leonards, J R & G Levy Absorption and metabolism of aspirin administered in enteric coated tablets *J Amer med Ass* 1965, **193** 99–104

Levy, G & L E Hollister Inter- and intrasubject variations in drug absorption kinetics *J pharm Sci* 1964a, **53**, 1446–1452

Levy, G and L E Hollister Failure of U S P disintegration test to assess physiologic availability of enteric coated tablets *N Y St J Med* 1964b **64**, 3002–3005

Levy, G & W J Jusko Case history of a pharmaceutical formulation failure *Clin Pharmacol Therap* 1967, **8**, 887–889

Lozinski, E Physiological availability of dicoumarol *Canad med Ass J* 1960, **83** 177–178

Orzack, M H, C L Taylor & C Kornetsky A comparison of the behavioural effects of periodic administration of chlorpromazine and a 'sustained release" form of chlorpromazine *Clin Pharmacol Therap* 1969 **10**, 258–264

Prescott, L F Pharmacokinetic drug interactions *Lancet* 1969, **2**, 1239–1243

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(XXV Scandinavian Pharmacological Meeting)

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ABSTRACTS

Volumen 29, Supplementum 4, 1971

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The abstracts of the free communications are placed alphabetically after the names of the speakers.

The indexes will help to find communications on similar subjects.

U. Abshagen[x], K.v. Bergmann, H. Kewitz and V. Rietbrock (Institut für Klinische Pharmakologie, 1000 Berlin 45, Hindenburgdamm 30) : ABSORPTION OF GLYCOSIDES IN RATS.

In biliary fistula rats receiving intraduodenally 40 uCi Digoxin (D), ß-Acetyldigoxin (AD) and ß-Methyldigoxin (MD) maximal blood levels were found after 60-180 min. The maximal blood concentration of D, AD and MD are related as 1:1.5:?.0. The halfe lives of D, AD and MD are 6.3; 7.5; 12.0 hours, respectively. The absorption rate can be taken from the time-course of the amounts of glycoside in the intestine. The curve shows two different exponential slopes. During the first two hours the k-values are 0.37 h^{-1}, 0.46 h^{-1} and 1.19 h^{-1}. During the following ten hours the k-values are 0.04 h^{-1}, 0.04 h^{-1} and<0.001 h^{-1} for D, AD and MD, respectively. In addition to these findings the estimation of glycosides in portal vein blood gave further proof, that the absorption of MD is almost completed within two hours, whereas the absorption of D and AD is carried on over the whole time of the experiment.
The values of absolute absorption rate after two hours are 52% for D, 60% for AD and 78% for MD, after 12 hours for D 66%, for AD 69% and for MD 81%.

Liisa Ah[illegible] ...ent of Pharmacology,
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Alcohol [illegible] BRAIN 5HT AND 5HIAA
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Lowering brain 5HT content by p-chlorphenylalanine reduces voluntary intake of ethanol in rats (Myers, R.D. & W.L. Veale: Science N.Y. 1968, 160, 1469-1471). Marked differences in voluntary alcohol consumption of rats become evident by selective outbreeding (Eriksson, K.: Science N.Y. 1968, 159, 739-741). Therefore brain 5HT and 5HIAA contents of male albino rats selected for 16 generations for their alcohol consumption were studied spectrophotofluorimetrically. Both 5HT and 5HIAA content in the brains of the alcoholic rats were 15 to 20 % higher than those of the non-alcoholic rats. After probenecid the difference in the 5HIAA content was 34 % (p<0.05). After the alcoholic rats had been a month in a free-choice situation their brain 5HT content was 31 % (p<0.001) and 5HIAA content 10 % higher than that of sober non-alcoholic controls. No changes were found in the brain 5HT and 5HIAA content of non-alcoholic rats who had been forced to drink the same amount of ethanol as alcoholic rats drank voluntarily (7.5 g/kg/day). The results further support the suggestion that cerebral 5HT metabolism may be involved in the selection of ethanol.

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Lowering brain 5HT content by p-chlorphenylalanine reduces voluntary intake of ethanol in rats (Myers, R.D. & W.L. Veale: Science N.Y. 1968, 160, 1469-1471). Marked differences in voluntary alcohol consumption of rats become evident by selective outbreeding (Eriksson, K.: Science N.Y. 1968, 159, 739-741). Therefore brain 5HT and 5HIAA contents of male albino rats selected for 16 generations for their alcohol consumption were studied spectrophotofluorimetrically. Both 5HT and 5HIAA content in the brains of the alcoholic rats were 15 to 20 % higher than those of the non-alcoholic rats. After probenecid the difference in the 5HIAA content was 34 % ($p<0.05$). After the alcoholic rats had been a month in a free-choice situation their brain 5HT content was 31 % ($p<0.001$) and 5HIAA content 10 % higher than that of sober non-alcoholic controls. No changes were found in the brain 5HT and 5HIAA content of non-alcoholic rats who had been forced to drink the same amount of ethanol as alcoholic rats drank voluntarily (7.5 g/kg/day). The results further support the suggestion that cerebral 5HT metabolism may be involved in the selection of ethanol.

F. Andreasen (Pharmacological Institute,University of Århus, Århus, Denmark): PROTEIN BINDING OF DRUGS IN SERUM FROM PATIENTS WITH ACUTE ANURIA:

The protein binding of six different drugs was investigated in serum from five normal persons and from ten patients suffering from acute anuria. In the normal material was found: acetylsalicylic acid 48.7±5.3%, sodium salicylate 8o.5±o.9%, phenylbutazone 89.3±1.6%, diphenylhydantoin 7o.4 ±1.9%, sulfadiazine 44.6±2.5%, thiopental 84.3±o.8%. In the patients (serum after hemodialysis): acetylsalicylic acid 22.6±8.6%, sodium salicylate 42.1±5.1%, phenylbutazone 69.9±7.1%, diphenylhydantoin 43.o±4.5%, sulfadiazine 17.7 ±4.6%, thiopental 61.9±5.2%. The protein binding was determined before and after the patients were hemodialysed and after a further dialysis of the serum in vitro. In the patients the following was found: before hemodialysis: acetylsalicylic acid -2.5±1.3%, phenylbutazone 74.9±o.9%, sulfadiazine 6.9±2.1%, after hemodialysis: acetylsalicylic acid lo.1±1.5%, phenylbutazone 81.9±o.6%, sulfadiazine 14.1 ±3.3%, after further dialysis: acetylsalicylic acid 18.o± 2.2%, phenylbutazone 85.6±o.5%, sulfadiazine 26.o±2.7%. The normal material, before dialysis: acetylsalicylic acid 58.4 ±1.1%, phenylbutazone 95.9±o.2%, sulfadiazine 44.9±1.2%, after dialysis: acetylsalicylic acid 58.1±1.o%, phenylbutazone 97.1±o.1%, sulfadiazine 49.4±1.o%. Experiments with serum ultrafiltrates confirmed that the findings could not be explained by the lower concentrations of protein.

E Änggård [x)], L.E. Jönsson and L.M. Gunne (Dpt of Pharmacology Karolinska Institutet, S 104 01 Stockholm and Psychiatric Res Centre, Ulleråker Hospital, S 750 17 Uppsala, Sweden)
PHARMACOLOGICAL BLOCKADE OF AMPHETAMINE EFFECTS IN SUBJECTS DEPENDENT ON CENTRAL STIMULANTS.

Euphoric effects were recorded by a self rating procedure after i.v. injection of 200 mg d,l-amphetamine sulphate in subjects dependent on central stimulants. Pretreatment with α-methyl-p-tyrosine (α-MT) 0.5 g and 1.0 g t i.d. decreased the magnitude of the self rated euphoric response by 50% and 75% respectively. After one weeks treatment with α-MT 1.0 g t.i.d. tolerance developed to the amphetamine blocking effect of α-MT. Following discontinuation of α-MT an enhanced response to amphetamine was observed. The plasma levels associated with a 50% blockade was dout 3 µg/ml of α-MT. Treatment with a single 4 g dose of α-MT gave a 70% blockade lasting for 24-36 hours. Of several neuroleptic drugs pimozide (20 mg) and chlorpromazine (50 mg t.i.d) gave a 50% and 40% reduction respectively of the amphetamine response. The results show that the euphoric effect caused by intravenous amphetamine is dependent on the presence of a small α-MT sensitive catecholamine pool in the brain It may be possible to use α-MT in suitably spaced intervals for the treatment of subjects dependent on the drug

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S -M Aquilonius[1] x, B Nyström[2], J Schuberth[3] and A Sundwall[4] ([1] Res. Inst Natl. Defence, Sundbyberg, [2] Dept. of Neurosurgery, Uppsala, [3] Psych Res Centre, Ulleråker Hospital, Uppsala and [4] Dept. of Pharmacology, Umeå Sweden) CEREBROSPINAL FLUID CHOLINE IN EXTRAPYRAMIDAL DISEASES

Using an enzymatic method (Jönsson, L E., Schuberth, J. & A. Sundwall Life Sci 1969, 8, 977-981) the concentration of the acetylcholine precursor and metabolite choline (Ch) was determined in cerebrospinal fluid (CSF from patients suffering from Parkinson's disease and Huntington's chorea. The following concentrations of Ch (nM/ml ± S.D) were found in lumbar CSF

Parkinson's disease	(n = 20)	2.6 ± 0.7
a) on anticholinergic drugs	(n = 10)	2 6 ± 0.4
b) no medication	(n = 4)	2 2 ± 0 6
c) 1-DOPA therapy	(n = 7)	1.7 ± 0 4
Huntington's chorea		
Controls (prostatectomia, cholecystectomia, tumor mam, minor surgery)	(n = 15)	2 4 ± 0.4

The finding of significantly lower CSF-Ch concentrations in Huntington's [illegible] on [illegible] o hyperfunction in striatal cholinergic mechanisms due to a disturbed dopaminergic inhibition on this region

P.Arvelo x) and N.T.Kärki (Department of Pharmacology, University o Oulu, Finland) EFFECT OF PHENOBARBITAL PRETREATMENT ON CERIUM INDUCED IMPAIRMENT OF DRUG METABOLISM IN RAT LIVER.

Cerium (Ce) impairs the oxidative drug metabolism in the rat (Arvelo, P. & N.T.Kärki Acta Pharmacol.Toxicol. 1970, 28, 36, suppl. 1). Recently we have investigated if phenobarbital (Phe) pretreatment effects the Ce induced changes in the drug metabolizing capacity of the liver. Male rats were injected i.v. with 2 mg of Ce/kg in 0.9 % saline. Another group was pretreated before Ce for ten days with Phe by replacing the drinking water with 0.05 % Phe solution. All animals were sacrified three days after Ce-injection. The livers were removed and homogenized in four volumes (w/v) of 0.1 M Tris-HCl buffer of pH 7.4 containing 1.15 % KCl. The 105 000 x g microsomal fraction was used for estimation of hexobarbital oxidase (HBO) uridine diphospho glucuronyl transferase (UDPGT) and 3,4-benzpyrene hydroxylase (BPH) activities. The cytochrome P-450 (Cyt P-450) content was determined from the microsomal and the glucose-6-phosphate dehydrogenase (G-6-P DHG) activity from the soluble fraction. The Phe pretreatment had a normalizing effect on the Ce induced changes in drug metabolism of rat liver. When the metabolic activities in the control series are taken as 100 % were the corresponding per cent values in Ce-treated, Phe-treated and simultaneously with Ce and Phe treated rats for HBO 34.0 %, 190.0 % and 154.0 %, for BPH 34.4 %, 135.5 % and 102.1 %, for Cyt P-450 40 0 %, 208.6 % and 140.0 % respectively. The Phe-treatment tends also to normalize the Ce-induced activating effect on UDPGT and G-6-P DHG and the corresponding values were 220.7 %, 589.7 %, 655.2 % and 189.6 %, 259.1 %, 233.4 % respectively.

J.BALDAUF (Inst.f.Pharmakol.& Toxikol., Homburg/Saar W.-Germany)
RELEASE OF A NEGATIVE CHRONOTROPIC AND DROMOTROPIC SUBSTANCE DURING CARDIAC HYPOXIA.

In isolated guinea pig hearts perfused according to Langendorff's technique hypoxia produces bradycardia, blockade of A-V-conduction and increased coronary flow. These effects are fully reversible and not blocked by atropine. Adenosine (10^{-6} to 10^{-5} g/ml) causes the same effects. It seems well established that adenosine is released from cardiac muscle during hypoxia (M.KATORI and R.M.BERNE, Circulation Research 19, 420 - 425 (1966)). The oxygenated perfusate collected during the period of hypoxic perfusion, however, failed to produce any effect on the measured parameters. Dipyridamole, which is able to prevent deamination of adenosine, was added in a concentration of 10^{-6} molar immediately after hypoxic perfusion to the collected perfusate. The perfusate was oxygenated and reperfused. In this case bradycardia, blocking of A-V-conduction and increased coronary flow were the same as during hypoxia or application of adenosine. Hence adenosine released by cardiac hypoxia may not only increase the coronary flow but also induce bradycardia and heart block.

E. Bechgaard (Royal Danish School of Pharmacy, Department of Pharmacology, DK-2100 Copenhagen, Denmark): ABSORPTION OF SALICYLIC ACID FROM THE PERFUSED HUMAN RECTUM.

An in situ technique for rectal perfusion in humans has been developed. An outer and an inner tube are placed in the rectum of a sitting experimental subject. The infusate is instilled through the outer tube. The solution rises to 8 cm in the rectum, where it is drained through the inner tube. This technique has been used to study the influence of conc. and pH on the absorption of salicylic acid. At a perfusion rate of about 7 ml/min. the conc. of salicylate in the effusate is approx. the same as that in the infusate. During the absorption period (15-45 min.) 4 blood samples are drawn. The plasma salicylate conc. plotted against time gives a straight line. The slope of this line is used as a measure of the absorption rate. The absorption from different solutions with conc.'s of 15.6-156 mM showed direct proportionality between absorption rate and conc. indicating that the membrane was not saturated. At salicylate solutions (47 mM) buffered to pH 8, 7, and 6 respectively, the relation between the absorption rates was about 1:2:3. According to the theory that only the lipid-soluble non-ionized form is absorbed, a relation of 1:10:100 should be expected.

B. Beermann[x], K. Hellström and A. Rosén (Clinical Pharmacology Laboratory and Department of Medicine, Serafimerlasarettet, 112 83 Stockholm, Sweden): THE GASTROINTESTINAL ABSORPTION OF ^{3}H-DIGOXIN IN MAN.

An aqueous solution of ^{3}H-12α-digoxin (0.25 mg, 50 uCi) and polyethylene glycol (PEG, nonabsorbable marker) was administered orally or by intrajejunal infusion to six healthy subjects. The ratio between the amount of radioactivity per mg PEG of gastrointestinal aspirates and that of the test solution demonstrated that 40-60 per cent of given label was absorbed in the proximal part of the digestive tract (50-200 cm from the nose). Considering that there was a 41-62 per cent yield of radioactivity in the urine during 14 days and that radioactivity excreted in the bile was presumably not fully reabsorbed, a minor amount of label was probably also taken up in the more distal parts of the gut. There was no evidence of a decomposition of ^{3}H-digoxin in the small intestinal aspirates. Most of the drug was excreted unchanged in the urine.

H.F. Benthe, G. Czok, A. Dwenger (Pharmakologisches Institut der Universität Hamburg, Bundesrepublik Deutschland) : REABSORPTION BILIARER ELIMINATIONSPRODUKTE VON DIGITOXIN UND DIGOXIN.

The metabolic products of cardiac glycosides in bile and urine may be divided into two main groups, one of which may be extracted with chloroform, the second being water soluble. In contrast to man the metabolism of digitoxin and digoxin in cats and guinea pigs yields preferably the water soluble products.

Acid hydrolysis of this water soluble fraction of bile and urine shows that conjugated products of the glycosides and genines exist as well as other metabolites changed in the genine molecule. The metabolites of the water soluble fraction behave quite different concerning their reabsorption and excretion after intraduodenal application in contrast to their behaviour after application of the native glycosides.

J.BALDAUF (Inst.f.Pharmakol.& Toxikol., Homburg/Saar W.-Germany)
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E Borsch* und K. Greeff (Pharmakologisches Institut der Universität 4 Düsseldorf, Moorenstr 5, Deutschland): EINFLUSS VON STEROIDEN AUF DIE STROPHANTHINEMPFINDLICHKEIT DER $NA^{+}+K^{+}$-STIMULIERTEN TRANSPORT-ATPASE.

Influence of sterolds on the sensitivity of the $Na^{+}+K^{+}$*-stimulated* ATPase against k-strophanthin Ergosterin and stigmasterin have no influence on the $Na^{+}+K^{+}$-stimulated membrane ATPase of guinea-pig heart muscle, but they antagonize in aequimolar concentrations (10^{-4}-10^{-5} M) the inhibitory effect of k-strophanthin by about 5o % or 2o % resp In comparison, cholesterin, desoxycorticosterone, testosterone, progesterone, cortisol and aldosterone (10^{-3}-10^{-6} M) are ineffective in vitro.
After a four days treatment of guinea-pigs (25 mg/kg) the inhibitory effect of k-strophanthin to the isolated membran ATPase is diminished half-maximale inhibition (mM k-strophanthin) was observed in controles o,o11, after desoxycorticosterone 1,75, testosterone o,3o, progesterone (o,16. The specific activity of the effect of k-strophanthin to en... is unchanged.

The toxicity of k-strophanthin (DL, mg/kg) is diminished after pretreatment untreated guinea-pigs o,96, after desoxycorticosterone 2,42, progesterone 1,96

U Breyer*) and D. Krauß (Institut fur Toxikologie, Universitat Tubingen, 74 Tubingen, and Organisch-chemisches Institut, Universitat Heidelberg, 69 Heidelberg, Germany)
ACCUMULATION OF METABOLITES DURING CHRONIC APPLICATION OF THE NEUROLEPTIC DRUG PERAZINE TO RATS.

Male rats were given 2 x 25 or 2 x 50 mg/kg perazine (methyl-piperazinyl-propyl-phenothiazine) daily per os. After various time intervals they were killed, and levels of perazine and its metabolites in several organs were determined by extraction and thin layer chromatography followed by UV spectroscopy. Besides desmethyl perazine, a novel metabolite was found to accumulate during treatment. The structure of this compound could be elucidated by mass spectrometry, IR and NMR spectroscopy. The metabolite has a remarkably long half-life in the rat organism, as could be shown by following the decline of its concentration in various organs after termination of the perazine treatment. Concomitant study of the elimination of perazine metabolites in urine led to the identification of a further degradation product.

V. Bollmann[x)] - . — . . al Division, Chem. W.
Albert, 6202 - 9101) THE INFLUENCE OF
METHYL-XANTHI - - - -- AGENTS ON ALPHA-ADRENERGIC
EFFECTS.

The influence of methylxanthines (BL 191 = 1-(5'-oxohexyl)-dimethylxanthine, SK 7 = 1-hexyl-3,7-dimethylxanthine, aminophylline) and ß-sympathomimetic agents (isoprenaline, buphenine) on the alpha-receptor as well as on the alpha-adrenergic effects of epinephrine respectively of norepinephrine was investigated on the isolated seminal vesicle of the guinea pig, on isolated aortic strips of rabbits and on the isolated spleen of the cat.

None of the investigated drugs except isoprenaline has shown direct alpha-adrenergic effects. Methylxanthines mainly inhibited the contractions caused by epinephrine or norepinephrine though some advers reactions have been seen. With isoprenaline the potentiating effect was prevalent, while buphenine was inhibiting the action of the catecholamines. The possibility of the inhibition of alpha-adrenergic effects by raised concentrations of cyclic 3',5'-AMP is discussed.

P. Bolme[x)], C. Sirtori and D. L Azarnoff (Clinical Pharmacology Toxicology Center, Departments of Medicine and Pharmacology, University of Kansas Medical Center, Kansas City, Kansas, USA) SOME METABOLIC EFFECTS OF L-DOPA IN PATIENTS WITH PARKINSON S DISEASE.

In 23 patients with Parkinson's disease venous blood samples were taken two, three and four hours after the administration of graded, oral doses of L-Dopa The samples were assayed for glucose, immunoreactive insulin (IRI), glycerol, free fatty acids (FFA) and human growth hormone (HGH). After 0 25 g of L-Dopa significant increases were seen in HGH at two hours and in FFA at four hours after administration A similar result was obtained following the 0.5 g dose. A one g dose of L-Dopa, after three months of treatment, elevated the levels of HGH and glucose at two hours, glycerol at three hours and FFA at four hours. The basal fasting levels of glucose, IRI, FFA, glycerol and HGH did not change during three months of L-Dopa administration. Since L-Dopa is converted to dopamine but only to a very small extent further to noradrenaline (Calne, D. B., F. Karoum, C. R. J. Ruthven & M. Sandler Brit J Pharmacol. 1969, 37, 57-68) it is suggested that the rise in blood glucose, glycerol and FFA levels was caused by an increase in peripheral dopamine This view is supported by the finding that L-Dopa in mice increased blood glucose and that this effect was blocked by inhibiting the conversion of L-Dopa to dopamine (Håkanson, R., I Lundquist & C. Rerup Europ J Pharmacol 1967, 1, 114-119)

F.W. Deckert[x] and K.P. Link (Department of Biochemistry, University of Wisconsin, Madison, Wis. 53706 U.S.A.):
PARTIAL FATE OF WARFARIN IN THE GUINEA PIG.

The metabolism of warfarin in the guinea pig was studied with respect to dose (1-100 mg/kg), time (12-hr intervals for 5 days), and animal age (1, 5 and 8 months old). For metabolic fate studies 1 and 2 mg/kg doses of 4-^{14}C-warfarin sodium were injected intraperitoneally into young male guinea pigs. Roughly 50% of the injected ^{14}C was excreted in the urine during the first 12 hrs, while 86% of the dose was found there within 7 days after injection; another 9% of the dose was observed in the feces during this period. Seven urinary metabolites were identified via inverse isotope dilution with an ether extract of the urine: Warfarin (W), 13%; 4'-OH-W, 28%; 6-OH-W, 4-5%; 7-OH-W, 2-3%; 8-OH-W, 1-2%; salicylic acid, 4%; and an intramolecular condensation product [2,3-dihydro-2-methyl-4-phenyl-5-oxo-γ-pyrano(3,2-c)(1)benzopyran], 4-5%. Approximately 40% of the urinary radioactivity remains unidentified. Chromatographic studies also indicated the presence of the above metabolites in feces, but in different relative amounts than found in the urine. At the 1-2 mg/kg dose level, no significant age effect could be detected in the gross excretion rate of metabolites. Injecting higher warfarin levels resulted in decreased initial excretion rates, but the total percentages excreted after 5 days was roughly the same. The individual metabolites fluctuated both with respect to dose levels and time. Since the metabolism of warfarin in the rat (W.M. Barker et al.: J. Pharmacol. Exp. Therap., 1970, 171, 307) has also been described, a species comparison will be included.

[illegible] and S.Blümcke (Medizinische [illegible]d Pathologisches Institut [illegible]):
[illegible] CONTENT AND UPTAKE OF THE LUNG.

In 4 dogs the content of enogenous and the uptake of tritiated noradrenaline (NA) in the normal and the reimplanted lung was determined 6-15-20 days and 6 years after the reimplantation. Surgery was performed by Nasseri, Eisele, Städtler and Bücherl, FU Berlin. The NA-content was determined fluorimetrically. 7-H3-DL-noradrenaline hydrochloride (125 uCi/animal, spec. activity 6.88 Ci/mMol) was injected intravenously 10 minutes before the animals were sacrificed.
In the short term expts. the NA-concentration of the arteries of normal lungs varied between 0.4 and 0.9 ug/g and decreased on the operated side to 0.1-0.3 ug/g. The H3-NA-uptake was diminished on the operated side to 1/5 to 1/10th of control values. The NA-concentration of normal bronchi was lower (0.08 to 0.3 ug/g) and seems not to be altered after reimplantation. The uptake of H3-NA was moderately diminished in the larger and enhanced in the smaller bronchi. There was no consistent change in the NA-concentration of lung tissue but a significant reduction of H3-NA uptake which, however, was not as pronounced as in arteries. Electron micrographs of the different lung structures will be correlated with these biochemical data.

O.J. Broch jr.[x] and H.C. Guldberg (Department of Pharmacology, University of Bergen, MFH-bygget, N-5000 Bergen, Norway): FEEDBACK CONTROL OF RAT STRIATAL DOPAMINE SYNTHESIS AFTER CATECHOL-O-METHYLTRANSFERASE INHIBITION.

The effect of inhibition of catechol-O-methyltransferase (COMT) by tropolone, 100 mg/kg i.p., on dopamine metabolism was studied in the rat striatum.

One hr after drug administration the concentration of dopamine (DA) increased to 115%, the O-methylated metabolites, 3-methoxytyramine(3-MT) and homovanillic acid (HVA), fell to 30% and the deaminated product, 3,4-dihydroxyphenylacetic acid (DOPAC), rose to 150% of normal. At 2.5 hr DA had fallen to 80% while 3-MT was normal and the HVA level remained low while DOPAC had returned to control values. All the values for the metabolites were normal at 24 hr.

Following a 20 min constant i.p. infusion of tyrosine-14C to control and tropolone pretreated rats the in vivo accumulation of striatal DA-14C, 3-MT-14C, HVA-14C and DOPAC-14C were determined. One and 2.5 hr after tropolone the radioactive label accumulated to only 10-20% of the control in the DA, 3-MT and DOPAC fractions. Labelled HVA was not found in any of the samples.

The results indicate that the main pathway for DA is through 3-MT to HVA. Inhibition of COMT results in a reduced synthesis rate for DA, probably, operating through a negative feedback inhibition by the increased level of DA. Moreover, the results indicate that O-methylation and deamination are not simple alternative pathways (Murphy, Robinson and Sharman: *Br. J. Pharmac.* 1969, 36, 107-115).

Sten Christensen (Department of Pharmacology, University of Copenhagen, Denmark): RENAL TUBULAR SECRETION OF RIBOFLAVIN IN THE RAT

The renal clearance of riboflavin and inulin-^{14}C-carboxylic acid was determined in anaesthetized Wistar rats during constant infusion of the compounds In the range of plasma riboflavin from 0 5 to 10 μg/ml, the ratio of riboflavin/inulin clearances varied individually from 1 2-3 2, indicating active tubular secretion of the vitamin Probenecid injected in large doses depressed the clearance ratio to 1 0 or slightly lower As some binding (21%) of riboflavin to plasma proteins was demonstrated in vitro, this indicates that back diffusion does not occur in the tubules. Precipitation of riboflavin in the kidneys at high plasma concentrations prevented the demonstration of a possible Tm for the tubular secretion The clearance ratio was generally unaffected by considerable variations of the GFR At very low plasma levels, however, the clearance ratio varied proportionally with the GFR and the urine flow. Partly on this basis is suggested that riboflavin is actively reabsorbed from the tubules by an easily saturable mechanism

S EDELFORS[x], I GØTHGEN. (University of Copenhagen, Department of Pharmacology, 20 Juliane Maries Vej DK 2100 Copenhagen Ø, Denmark) DISTRIBUTION OF ELECTROLYTES WITHIN THE BRAIN IN LITHIUM TREATED RATS.

Rats were treated with lithium orally for two or five weeks (serum-lithium 0,5 mMol/l on the time for examination) The distribution of lithium within the brain and the influence of the treatment upon the water/dry matter-ratio and the potassium- and sodium contents were examined The water intake per day increased throughout the initial four weeks (from 20 ml to about 100 ml) The concentration of lithium was found to be two to four times greater in the hypothalamic region than in other parts of the brain, and at the same time four to six times greater in the white than in the grey matter The water/dry matter-ratio decreased in all parts of the brain, except the hypothalaamus, by approximately 50 % The total contents of potassium and sodium were unaltered

L.EDELMANN and K.PFLEGER (Inst.f.Biophysik - Boris Rajewsky-Inst. - Inst.f.Pharmakol.& Toxikol. Univ. d. Saarlandes, Homburg/Saar W.-Germany) POTASSIUM AND CESIUM EXCRETION BY KIDNEY AND GUT OF RATS.

In earlier reports (L.EDELMANN, K.PFLEGER and K.-H.MATT, Biophysik 7, 181 (1971), L.EDELMANN, Biophysik 7 (1971) in press) it was demonstrated that the well known preferred accumulation of cesium 137 compared with potassium in mammalian organism is caused by different kinetics of these alkali ions. This different behaviour could be described by means of a mathematical model which postulated a relative rapid excretion of cesium by kidney and gut. New experiments on rats showed an excretion ratio Cs/K of 2 3, when the plasma ratio was 1 1. Compared with the transport rates of cesium and potassium between plasma and other organs - where cesium is transported very slowly compared with potassium (EDELMANN et al., Biophysik 7, 181 (1971)) - this excretion ratio Cs/K 2 3 means in fact a relative quick excretion of cesium. The greatest amount of cesium (85 %) and potassium (93 %) was excreted by the kidney. From the excretion ratio and the relative amounts in urine and faeces results $(Cs/K)_{urine} \approx 1\ 1.64$ and $(Cs/K)_{faeces} \approx 1\ 0\ 7$. The accumulation ratio Cs/K was found in the kidney to be 2.5 1 and in the gut 1.5 1. From these values can be calculated the excretion ratios $(Cs/K)_{urine}/(Cs/K)_{kidney} \approx 1\ 4$ and $(Cs/K)_{faeces}/(Cs/K)_{gut} \approx 1\ 1$.

W Dünges[x)] and K J Netter (Pharmakologisches Institut der Universität, D 6500 Mainz, Germany) ON THE GAS CHROMATOGRAPHY OF HYDROXYLATION PRODUCTS OF ANISOLES

Microsomes were prepared from rabbit livers and suspended in a phosphate buffer, containing KCl, $MgCl_2$ and a NADPH regenerating system In 1 ml of this suspension various anisoles (0 2 to 0 5 µmoles) were incubated for different time periods. The incubate was extracted with a purified organic solvent. Using a special micro technique (Beyermann, K., A.Kessler & P.W.Ungerer Zeitschr.f.Anal.Chemie, 1970, 25, 289-293), which minimizes the evaporation of the solutes, the extract was concentrated from 1 ml to 0.005 ml. With other concentrating procedures a drastic loss of solutes occurs when the volume is reduced below 0.1 to 0.05 ml. Without further steps of separation 0.2 to 1.0 µl of the solution were injected into the gas chromatograph. The direct gas chromatography avoids a loss of volatile solutes which is marked when the gas chromatographic analysis is preceded by another separation method, e.g. thin layer chromatography (Daly, J., Biochem.Pharmacol., 1970, 19, 2979-2993).

The advantage of the method is obvious from a high recovery rate of unchanged anisoles and their hydroxy metabolites which therefore can be estimated simultaneously in micro amounts after incubation.

R Ebert[*)] and U Schwabe (Institut für Pharmakologie, Medizinische Hochschule, 3000 Hannover, Roderbruchstr 101, Germany) ANTILIPOLYTIC EFFECT OF ADENOSINE AND PURINE BASES IN ISOLATED FAT CELLS

The effect of nucleosides and purine bases on lipolysis induced by theophylline, norepinephrine and dibutyryl cAMP was investigated in isolated fat cells of rat adipose tissue Adenosine was the most potent antilipolytic agent of all nucleosides and purine bases tested Adenosine (5 µM) completely blocked lipolysis induced by theophylline (0,5 mM), but only partially (40 %) inhibited lipolysis induced by norepinephrine (1 µM) The inhibitory potencies of adenosine were antagonized by increasing concentrations of theophylline (but not of norepinephrine), indicating a competitive antagonism between theophylline and adenosine In contrast, lipolysis induced by dibutyryl cAMP (1 mM) was enhanced by adenosine (5 µM) Similarly, basal lipolysis was stimulated by adenosine Inosine, hypoxanthine, xanthine and guanosine in high concentrations (0,1 - 1 mM) had a weak inhibitory effect on norepinephrine- and theophylline-induced lipolysis These results suggest, that the antilipolytic action of adenosine is produced by adenosine itself or by phosphorylated adenine nucleotides, which are synthetized from adenosine within the fat cell

L. Finch [x)] and G. H. ... of
F. Hoffmann - La Roc

VASCULAR RESISTANCE ...

Recent studies with the perfusion of the hindquarters (Folkow, B. et al.: Acta physiol. scand. 1970, 80, 93-106) or the entire vascular bed (Folkow, B. et al.: Acta physiol. scand. 1970, 79, 373-378) indicated in genetically hypertensive rats (GHR) an increased resistance to flow as compared with normotensive rats (NR). In the current study, perfusions of the entire vascular system (with the exception of the pulmonary and coronary vascular bed) and of isolated hindquarters were carried out under conditions of maximal vasodilation in NR and several types of hypertensive rats. Both renal (RHR) and desoxycorticosterone/saline hypertensive rats (DSHR), but not GHR, showed an increased resistance in the whole vascular bed. Similar results were obtained when only the isolated hindquarters of the rats were perfused. Using the latter preparation an increased maximal vasoconstrictor response to noradrenaline (NA) and 5-hydroxytryptamine was found in RHR and DSHR as compared to NR. In these two types of hypertensive animals the threshold vasoconstrictor dose of NA was lower than in NR. GHR differed insofar as the maximum of only the NA dose-response curve was elevated and that the threshold doses for both agonists were identical with those of NR. The different values of vascular resistance and the different shapes of the dose-response curves for vasoconstrictor agents in several types of experimental hypertension make it improbable that in all cases structural changes of the vessel wall are the sole reason for the elevated blood pressure.

B. Fjalland[x)] and V. Pedersen (Department of Pharmacology, H. Lundbeck & Co. A/S, Copenhagen, Denmark): ON THE ROLE OF BIOGENIC AMINES IN THE CONTROL OF GASTRIC SECRETION IN RATS.

The influence of biogenic amines on the control of gastric secretion has been investigated using pylorus-ligated rats (Shay-rats).

The tyrosine hydroxylase inhibitor, H 44/68 (d,l-α-methyl-tyrosine-methylester hydrochloride, 50 mg/kg i.p. 25 h -, 100 mg/kg i.p. 18 h - and 50 mg/kg i.p. 2 h before test) increased volume and acid output, the effect being strongly inhibited by atropine. L-Dopa (200 mg/kg s.c. 1 h before test) partly inhibited the effect produced by H 44/68.

The tryptophane hydroxylase inhibitor, d,l-p-chlorophenyl-alanine-methylester hydrochloride (PCPA), 100 mg/kg i.p. in three days prior to test inhibited volume and acid secretion. Treatment with 5-HTP 50 mg/kg i.p. 1 h before test partly restored the volume and acid output decreased by PCPA. H 44/68 given to rats pretreated with PCPA resulted in an increase in volume and acid secretion, the output being about normal control level.

The results indicate the possibility of a 5-HT - NA/DA balance in control of gastric secretion in rats.

S.Ahlenius and J.Engel[x] (Department of Pharmacology, Fack, S-400 33, Gothenburg 33, Sweden): POTENTIATION OF HALOPERIDOL BY TYROSINE HYDROXYLASE INHIBITION.

Drugs of the phenotiazine and butyrophenone group have been shown to depress different kinds of human and animal behavior. These effects are thought to be connected with the ability of these drugs to block post-synaptic receptors in the central catecholamine neurons, resulting in an increased catecholamine turnover (Andén, N-E., Carlsson, A. and J. Häggendal: Ann. Rev. Pharmac. 1969, 9, 119-134). The effect of haloperidol, a butyrophenone derivative, after pretreatment with H 44/68, a tyrosine hydroxylase inhibitor, on food-reinforced operant behavior (fixed ratio 40:1) was investigated. The intraperitoneal injections of haloperidol in doses (≤0.05 mg/kg) that had no effects *per se*, resulted in a marked depression of the behavior studied when given after subthreshold doses of H 44/68 (≤50 mg/kg). The obtained results are indicative of a mechanism of potentiation and are discussed in terms of a feed-back mechanism connecting the pre- and postsynaptic neurons.

P.Ertama[x], P.Ylitalo and L.Ertama (Department of Pharmacology, University of Helsinki, Helsinki, Finland): TOXICITY OF SOME VASOACTIVE DRUGS AND THEIR EFFECT ON RETINAL VESSELS IN ANAESTHETIZED RATS.

The aim of this investigation was to study the possible central vasoconstriction as an ethiology in the highly increased intravenous [illegible] esthesia in rats (Tammisto,T.: [illegible], suppl.7). For this purpose LD [illegible] (NA), adrenaline (A), lypressin (LP), argipressin (AP) and bradykinin (BK) were determined in conscious and anaesthetized rats (pentobarbital 40 mg/kg intraperitoneally). In order to estimate central vasoconstriction photographs of retinal vessels in anaesthetized rats were taken after intravenous injection of sublethal doses of 5HT, NA, A, LP and BK. The LD_{50}-values (mg or IU/kg) were a) awake, b) in anaesthesia: 5HT a) 60±8,5, b) 0,1±0,01 (Tammisto, 1965); NA a) 0.14±0,03, b) 0,020±0,005; A a) 0,06±0,01, b) 0,020±0,005; LP (three LD_{50}-values) a) 5,4±1,0, 7,2±2,6, 170±10, b) 5,4±1.0, 110±20, 200±30; AP a) 200±20, b) 130±20; BK a) not performed, b) >4. None of the studied agents caused vasoconstriction in the retinal vessels. The toxicity of used agents was not increased similarly as that of 5HT. The previous observation of retinal vasoconstriction after intravenous 5HT in rats (Tammisto, 1965) could not be verified. The results do not favour the idea of central vasoconstriction as an explanation for the increase of toxicity of 5HT in anaesthetized rats.

K.Fuxe, " . . . . sedman and B.Anagnoste
(Departm . . . . . . Institutet, Stockholm,
Sweden a . . . New York Medical Cen-
ter, New . . . . . .)F HYPOPHYSECTOMY AND
ACTH TRE... . . . . . . THESIZING ENZYMES IN
TISSUES.

With the use of immuno-fluorescence techniques and with specific radioisotope methods changes in tyrosine-hydroxylase (TH), DA-β-hydroxylase (DβH) and in phenylethanolamine-N-methyl-transferase (PNMT) have been studied in adrenal glands, blood and brain after hypophysectomy. The immunohistofluorescence studies demonstrated that practically no specific PNMT immuno-fluorescence remained in the A containing cells of the adrenal medulla following hypophysectomy. Also the specific DβH immuno-fluorescence in the A and NA gland cells was reduced in the hypophysectomized rats. In agreement with previous works it was found that the TH, DβH and PNMT activity was reduced following hypophysectomy and partly restored following ACTH treatment in the adrenal medulla. However, the blood levels of DβH were markedly increased following hypophysectomy, and these were reduced by ACTH treatment. Castration or adrenalectomy did not induce any changes in blood DBH levels. These results together with previous findings show the importance of the pituitary adrenal axis in the CA synthesis in the adrenal medulla. Furthermore, the data indicate that the pituitary gland can control the activities of DβH in sympathetically innervated tissues.

G. Fülgraff[x)], D. Sudhoff and H. Nünemann (Abt. Pharmakologie der Med. Fakultät, Medizinisch-Theoretische Institute der TH, 51 Aachen, Germany) EFFECTS OF FUROSEMIDE ON RENAL GLUCONEOGENESIS IN RATS AND DOGS IN VITRO.

Furosemide is a potent inhibitor of renal tubular sodium reabsorption. Renal Oxygen consumption, however, is not affected in vivo. Nevertheless, furosemide inhibits renal net uptake of lactate in dogs in vivo without influencing renal glucose balance. In the present studies the in vitro effect of furosemide on cortical gluconeogenesis from various precursors has been investigated. Slices of cortical tissue of rats and dogs were incubated for 60 min in Krebs-Ringer bicarbonate buffer with 10^{-2} M substrate at 38° C. If furosemide was added at concentrations of 10^{-4} M, glucose production was unimpaired with malate or α-ketoglutarate as substrates. It was decreased with lactate or pyruvate and increased with fructose as substrates. The same results were obtained, if furosemide was injected into the animals 20 min prior to the removal of the kidneys but was omitted from the incubation medium. Slices from renal medulla of dogs were incubated in hypertonic Krebs-Ringer solution with 10^{-2} M glucose as substrate. Furosemide 10^{-4} M increased significantly production of lactate and pyruvate. The results suggest that furosemide stimulates glycolysis in the renal medulla and decreases cortical utilisation of lactate and pyruvate for gluconeogenesis.

K.F. Franck (Dept. of Pharmacology, H. Lundbeck & Co. A/S, Copenhagen, Denmark): ORDER OF POTENCY OF SOME THYMOLEPTIC DRUGS AS BASED ON RESERPINE SYNERGISM IN THE FROG.

Thymoleptic drugs enhance the effects of reserpine (10 mg/kg i.m.) in frogs pretreated with the monoamine oxidase inhibitor phenelzine (25 mg/kg i.m.). This enhancement probably reflects activation of central 5-hydroxytryptamine-(5-HT)-dependent processes and it might be correlated with clinical mood-elevating effect (Lapin, I.P., Oxenkrug, G.F., Osipora, S.V. & N.V. Uskova: J. Pharm. Pharmacol. 1970, 22, 781-782). Reserpine synergistic effects of imipramine (IMI), desipramine (DMI), chlorimipramine (CIMI), amitriptyline (ATL), nortriptyline (NTL), chlorinated indane derivative Lu 8-052, thiophthalane derivative Lu 5-003 and chlorpromazine (CPZ) were studied in phenelzine pretreated frogs (Rana temporaria). The doses used ranged from 0.01-160 mg/kg i.m. The frogs were placed upside down and behavioural changes were observed. The order of potency of drugs causing loss of righting reflex (sedation) and of drugs producing twitches of the extremities was CIMI ≫ ATL ≥ IMI > DMI = NTL ≥ Lu 8-052 ≫ Lu 5-003 > CPZ and CIMI ≫ IMI ≥ ATL > NTL ≥ DMI = Lu 8-052, respectively. CPZ and Lu 5-003 did not produce twitches. The appearance of twitches in particular may be considered an index of central 5-HT potentiation since the order of potency of the twitch-producing imipramine-like drugs is in positive correlation with their order of potency as regards the known 5-HT membrane pump inhibition (Carlsson, A.: Bayer-Symposium II, 1970, 223-233)

M. Frisk-Holmberg (Department of Pharmacology, Karolinska Institutet, S-104 01, Stockholm 60, Sweden): THE INTERFERENCE OF TRICYCLIC PSYCHOACTIVE DRUGS ON THE UPTAKE OF BIOGENIC AMINES BY ISOLATED MAST CELLS.

Rat mast cells in vitro are capable of accumulating 5-hydroxytryptamine by an energy requiring process, (Jansson 1970) and histamine by diffusion (Cabut and Haegermark 1966). Tricyclic psychoactive drugs (chlorpromazine, derivatives and antidepressant drugs) interfere with the uptake of biogenic amines. In the present study the uptake mechanisms of 5-hydroxytryptamine (5-HT), dopamine (DA), noradrenaline (NA) and histamine (Hi) and the interference of these drugs were investigated. Rat mast cells were incubated with ^{14}C-labelled amine for 10 min. washed and the radioactivity measured in final sediments. Drugs were added to cells before labelled amines. The uptake of 5-HT and DA was saturable at low extracellular amine concentrations (< 50 µM) and could be described by a Lineweaver-Burke representation of the Michaelis-Menten kinetics. NA uptake was saturable at higher concentrations (< 200 µM) but the Hi uptake was not saturated within the concentrations range used (< 400 µM). The uptake rates of the saturable uptake processes were reduced by metabolic inhibitors and increased by increasing temperature, stimulated by Ca^{++}. It is suggested that the uptake of 5-HT and DA primarily involved an active transport, whereas the uptake of NA preferentially operates by facilitated diffusion and Hi by diffusion. Chlorpromazine in concentrations 10^{-6} - 10^{-5} M, competetively blocked the uptake of 5-HT and DA and reduced the uptake rates of the other amines. The drugs investigated had different affinities to the uptake sites. It is concluded that mast cells accumulate amines by active transports mechanisms and diffusion and that tricyclic psychoactive drugs interfere with both

K.Fuxe, M.Goldstein, T.Hökfe
(Department of Histology, Ka
Sweden and Department of Psy
ter, New York, U.S.A.): THE
ACTH TREATMENT ON CATECHOLAM... -
TISSUES.

With the use of immuno-fluorescence techniques and with specific radioisotope methods changes in tyrosine-hydroxylase (TH), DA-β-hydroxylase (DβH) and in phenylethanolamine-N-methyl-transferase (PNMT) have been studied in adrenal glands, blood and brain after hypophysectomy. The immunohistofluorescence studies demonstrated that practically no specific PNMT immuno-fluorescence remained in the A containing cells of the adrenal medulla following hypophysectomy. Also the specific DβH immuno-fluorescence in the A and NA gland cells was reduced in the hypophysectomized rats. In agreement with previous works it was found that the TH, DβH and PNMT activity was reduced following hypophysectomy and partly restored following ACTH treatment in the adrenal medulla. However, the blood levels of DβH were markedly increased following hypophysectomy, and these were reduced by ACTH treatment. Castration or adrenalectomy did not induce any changes in blood DBH levels. These results together with previous findings show the importance of the pituitary adrenal axis in the CA synthesis in the adrenal medulla. Furthermore, the data indicate that the pituitary gland can control the activities of DβH in sympathetically innervated tissues.

G. Fülgraff[x], D. Sudhoff and H. Nünemann (Abt. Pharmakologie der Med. Fakultät, Medizinisch-Theoretische Institute der TH, 51 Aachen, Germany) EFFECTS OF FUROSEMIDE ON RENAL GLUCONEOGENESIS IN RATS AND DOGS IN VITRO.

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B.Fyrö, H.Nyback and G.Sedvall (Departments of Psychiatry, S:t Görans Hospital, and Pharmacology, Karolinska Institutet, Stockholm, Sweden)

EFFECTS OF NIGRAL LESION AND CHLORPROMAZINE TREATMENT ON TYROSINE HYDROXYLASE ACTIVITY IN CORPUS STRIATUM OF THE RAT

Tyrosine hydroxylase activity in sucrose extracts of rat corpus striatum was determined by measuring the formation of tritiated water from 3,5-3H-L-tyrosine according to Mueller et al. (Mueller, R.A., H. Thoenen & J.Axelrod: J.Pharmacol. Exp. Ther. 1969, 169, 74-79). Rats were subjected to unilateral stereotaxic lesions in the crus cerebri at the level of the corpus mamillare where the nigro-neostriatal dopamine pathway is known to pass. Four days after the operation, the activity of tyrosine hydroxylase as well as the content of dopamine in corpus striatum on the side of the lesion was reduced to about 10 per cent of that of the intact side. Following acute and chronic treatment with chlorpromazine, the synthesis of 14C-dopamine from i.v. administered 14C-tyrosine was accelerated severalfold (Nyback,H. and G.Sedvall: J.Pharmacol. Exp. Ther. 1968, 162, 294-301). The tyrosine hydroxylase activity in the corpus striatum was not significantly altered following acute or chronic chlorpromazine treatment. The results indicate that chlorpromazine accelerates dopamine synthesis in the corpus striatum by other mechanisms than by altering the level of tyrosine hydroxylase in the tissue.

K.K. Gauri, R.D. Walter (Pharmakologisches Institut der Universität Hamburg, Bundesrepublik Deutschland):

5-ETHYL-2'-DEOXYURIDINE: INHIBITION OF DEOXYTHYMIDINE KINASE ACTIVITY.

We have shown that 5-ethyl-2'-deoxyuridine (EDU) is a potential inhibitor of DNA viruses like Herpes and Vaccinia. Also it has been shown to be incorporated into the DNA of rabbit cornea cells, Hela cells, Herpes-virus as well as into that of the E.Coli phage T_3. In spite of this incorporation EDU is neither mutagenic to the above mentioned Coli phage T_3 nor to a strain of Drosophila melanogaster. These results indicate that the virostatic effect of EDU is not essentially a function of its incorporation into the DNA. The possibility that the virostatic activity might be related to an inhibition of some enzymes concerned with the DNA synthesis may not be ruled out.
In the present paper we have examined the effect of 5-ethyl-2'-deoxyuridine on the primary key enzyme deoxythymidine kinase. The inhibitory results with EDU and some higher homologue 5-alkyl-2'-deoxyuridines will be reported.

B.Geierhaas und K.Flemming (Heiligenberg-Institut,7799 Heiligenberg und Radiol.Inst.d.Univ.,78 Freiburg i.Br., BRD):

DIE WIRKUNG VON CYSTEAMIN AUF DIE NEBENNIEREN-CORTICOSTEROIDE VON RATTEN.

Ausgehend von den Befunden, dass der 2,5 Std. nach Ganzkörperbestrahlung auftretende Corticosteronanstieg bei Ratten durch Cysteamin unterdrückt wird (K.Flemming und B.Geierhaas: Naturwiss.1967,18,493-494), untersuchten wir, ob auch die durch andere Agentien verursachten Corticosteronanstiege durch Cysteamin beeinflusst werden.
Die Corticosteronanstiege 30 Min.nach i.p.Injektion von Natriumsalicylat, 300 mg/kg und Histamin, 7,5 mg/kg wurden ebenso gehemmt wie die nach einer kurzdauernden Äthernarkose oder 2,5 Std. nach Injektion von 50 µg/kg u.250 µg/kg Bakterientoxin. Die Cysteamindosis betrug 150 mg/kg und wurde 120 bis 150 Min. vor dem Stimulantien der Nebennierenrinde (NNR) injiziert.
Versuche mit ACTH,5E/kg ergaben, dass Cysteamin nicht auf die übergeordneten Zentren der NNR, sondern auf die NNR selber einwirkt. Dünnschichtchromatografische Auftrennungen der Nebennieren-Extrakte zeigten, dass Cysteamin das 11ß-Hydroxylasesystem der NNR-Mitochondrien stört. Dadurch kommt es trotz maximaler Stimulation des Organs nicht zu einem Corticosteronanstieg. Die Corticosteronwerte der mit Cysteamin behandelten Ratten blieben im Kontrollbereich, während die Werte für 11-Desoxycorticosteron stark anstiegen.

BITION

THROMBOCYTES

Rat thrombocytes were incubated in plasma at 37° for 30 minutes in

20-32%, at 10 meq /l 46-50% and at 20 meq /l 63-78% Thrombocytes from rats treated with lithium for 2-55 days were incubated with 5-HT 1 7 x 10^{-5} M At no time were there any difference between the lithium treated and the control animals

Gerhard Gennser[x], Ingmar Lundquist and Erik Nilsson (Department of Pharmacology, and Department of Obstetrics and Gynaecology at the General Hospital of Malmo, University of Lund, Lund, Sweden):
DEVELOPMENT OF GLYCOGENOLYTIC ACTIVITY IN HUMAN FOETAL LIVER

Activities of glycogenolytic enzymes, α-glucan phosphorylase, glucose--6-phosphatase, and acid amyloglucosidase were investigated in human foetal liver, 13-23 weeks gestational age. In addition levels of liver glycogen and intrahepatic glucose were measured. Phosphorylase and glucose-6-phosphatase activities did not vary with foetal age, and were both 10-20% of the activities in adult liver. Acid amyloglucosidase activity and liver glycogen content increased within the development period studied and reached adult values already around the 20th week. A significant increase in intrahepatic glucose content and acid amyloglucosidase activity was found after a short period of foetal asphyxia. No change in glycogen content or the activities of phosphorylase and glucose-6-phosphatase was recorded after this intervention. The results indicate an important role of the hydrolytic as compared to the phosphorolytic breakdown of glycogen in human liver during early foetal stage.

I. Gøthgen[x], S. Edelfors. (University of Copenhagen, Department of Pharmacology, 20 Juliane Maries Vej DK 2100 Copenhagen Ø., Denmark): INVESTIGATIONS ON THE ELECTROLYTE CONTENTS OF ANATOMICALLY DEFINED PARTS OF THE BRAIN IN NORMAL AND LITHIUM-TREATED RATS.

The electrolyt contents of small and anatomically well--defined parts of the brain were examined. By the applied technique of dissecting reproducible results were obtained with very little biological variation. On basis of the results emphasis is put to the advantage of expressing the results in "total amounts per the various brain-parts" instead of the more commonly used relative figures.

E. Habermann (Pharmakologisches Institut, Rudolf Buchheim-Str. 4, Giessen, Germany):

DISTRIBUTION OF ^{125}J TETANUS TOXIN WITH AND WITHOUT ANTITOXIN, AND OF ^{125}J TOXOID UPON LOCAL APPLICATION IN RATS.

Sublethal doses of ^{125}J-labelled tetanus toxin (16,7 - 25 ng/kg; about 12 000 cpm/kg) were injected into the m.gastrocnemius of rats; antitoxin (500 U/kg) was injected at various times thereafter into another leg. Radioactivity of the various segments of spinal cord, sciatic nerves, leg and blood plasma were determined after 1,2,4,7,13 and 24 days. Results: 1. The degree of local tetanus does not run parallel with the radioactivity present in the lumbal part of the spinal cord. 2. Radioactivity persists there for the whole experimental period. 3. Simultaneous application of antitoxin prevents the spinal accumulation of radioactivity. Given 10 hours later, antitoxin diminishes the spinal radioactivity considerably; when it is applied 48 after the toxin, the spinal radioactivity is only slightly influenced. 4. The level of radioactivity in blood plasma is higher in animals with than without antitoxin. 5. Toxoid is also accumulated by the spinal cord; however, higher amounts must be applied compared with toxin. - The results yield a pharmacokinetic explanation for the long duration of local tetanus and for the restricted efficiency of antitoxin. They suggest that spinal binding of tetanus toxin is to be differentiated from its spinal efficacy.

K.-J. Hahn*) and E. Weber (Medizinische Universitatsklinik, Abteilung für klinische Pharmakologie, 69 Heidelberg,FRG):

EFFECT OF CHOLESTYRAMINE ON THE GASTROINTESTINAL ABSORPTION OF PHENPROCOUMAROL AND ACETYLSALICYLIC ACID.

In patients with hypercholesterolemia suffering from coronary disease, myocardial infarction or having had coronary surgery we encountered difficulties to establish effective low prothrombin levels with phenprocoumarol at simultaneous administration of cholestyramine. Determinations of plasma levels of phenprocoumarol in patients and volonteers yielded a considerable inhibition of the absorption of phenprocoumarol under concomitant administration of cholestyramine and consequently a diminished effect on prothrombin time. Acetylsalicylic acid was studied because of its use in the prophylaxis of thrombosis. The absorption of acetylsalicylic acid mainly was delayed by cholestyramine without great effect on the total amount absorbed. The results are in accordance with determinations of the binding of the two drugs to cholestyramine in vitro.

Gerhard Gennser[x)], Ingmar Lundquist and Erik Nilsson (Department of Pharmacology, and Department of Obstetrics and Gynaecology at the General Hospital of Malmö, University of Lund, Lund, Sweden).
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I. Gøthgen[x], S. Edelfors. (University of Copenhagen, Department of Pharmacology, 20 Juliane Maries Vej DK 2100 Copenhagen Ø., Denmark): INVESTIGATIONS ON THE ELECTROLYTE CONTENTS OF ANATOMICALLY DEFINED PARTS OF THE BRAIN IN NORMAL AND LITHIUM-TREATED RATS.

The electrolyt contents of small and anatomically well--defined parts of the brain were examined. By the applied technique of dissecting *reproducible results were obtained* with very little biological variation. On basis of the results emphasis is put to the advantage of expressing the results in "total amounts per the various brain-parts" instead of the more commonly used relative figures.

[illegible] J.Knoll(Department of Pharma- [illegible] iversity, Budapest, Hungary): [illegible] HOXYPHENYLETHYLAMINE (PMPEA), [illegible] (DMPEA) AND p-BROMOMETH- [illegible]

Amphetamines substituted in para-position e.g. PEMA have a LSD (mescaline)-like effect in animals (J.Knoll, E.S. Vizi and Z.Ecseri: Arch.Int.Pharmacodyn.1966,159,442-451). Development of tolerance to PEMA and cross-tolerance between this and LSD was demonstrated (J.Knoll and E.S.Vizi: Pharmacology 1970, 4, 278-286). However, DMPEA is believed to play some role in mental disturbances, and it was found that it has some LSD-like effect in animal experiments. Therefore we studied the effects of DMPEA and PMPEA both substituted in para-position. Both compounds exerted an inhibitory effect on escape behaviour of the rat, in the modified jumping test (J.Knoll and B.Knoll: Arch.Int. Pharmacodyn. 1964, 148, 200-216). ED50 for DMPEA is 150 mg/kg s.c, and for PMPEA 45 mg/kg s.c. However these values were reduced when the animals were pretreated with iproniazid. PEMA (15 mg/kg s.c. for 14 days) which otherwise reduced the 5-HT content of rat brain by 60% was able to prevent the inhibitory effect of DMPEA and PMPEA on escape behaviour, and there was now seen an exitatoric effect. The LD50 values were not changed. p-Chlorophenylalanine (100 mg/kg i.p. for three days) also prevented the effect of DMPEA and PMPEA on escape behaviour.

J. Holm (Department of Pharmacology, University of Copenhagen, Denmark): TETRAETHYLAMMONIUM UPTAKE BY MOUSE KIDNEY SLICES.

Recent studies suggest that decamethonium and tetraethylammonium (TEA), a marker substance for the *organic cation* secretion mechanism in the kidney, share a common transport mechanism in mouse kidney slices (Holm, J.: Acta pharmacol. et toxicol. 1971, in press). The uptake of TEA was studied for the purpose of comparing it to that of decamethonium. Kidney slices were incubated in Krebs-Ringer bicarbonate medium (pH 7.4, 37°) containing 14-C-TEA, and the slice-to-medium concentration ratio (S/M ratio) was measured. TEA (2 µM) was rapidly accumulated in oxygen-carbondioxide (95:5) atmosphere, and steady state with S/M ratio approximating 20 was obtained within ½ hr. Radioactive material extracted corresponded chromatographically to authentic TEA. S/M ratio was significantly reduced in the presence of metabolic inhibitors or compounds possessing positively charged nitrogen atoms, such as decamethonium, carbacholine, choline, hemicholinium-3, d-tubocurarine and atropine. S/M ratio decreased when raising TEA concentration. In nitrogen-carbondioxide (95:5) atmosphere S/M ratio (2 µM) reached a constant value around 1 within ½ hr., and did not decrease when raising TEA concentration. It is concluded that TEA is concentrated in mouse kidney slices as the unchanged compound. The uptake shows saturation, requires energy and is inhibited by structure analogues.

R Håkanson and G Liedberg (Departments of Pharmacology and Surgery, University of Lund, Lund, Sweden) EVIDENCE AGAINST HISTAMINE AS THE FINAL CHEMOSTIMULATOR OF GASTRIC ACID SECRETION

Vagal excitation by insulin hypoglycemia causes release of gastric histamine, activation of histidine decarboxylase, and stimulation of acid secretion in the normal rat After antrectomy, insulin stimulates acid secretion but is without effect on either gastric histamine levels or histidine decarboxylase activity Thus, insulin-induced stimulation of acid secretion occurs independently of histamine release or histamine formation, indicating that histamine is not a mediator of the vagal effect Hexamethonium prevents histamine-induced stimulation of gastric acid secretion but not that induced by pentagastrin. Histamine is consequently not a necessary intermediate in the pentagastrin--induced stimulation of acid secretion

R Håkanson and F Sundler (Departments of Pharmacology and Histology, University of Lund, Lund, Sweden) FORMALDEHYDE CONDENSATION A METHOD FOR THE FLUORESCENCE MICROSCOPIC DEMONSTRATION OF PEPTIDES WITH N-TERMINAL TRYPTOPHAN RESIDUES

N-Terminal tryptophan dipeptides, enclosed in a dried protein matrix, were found to react with formaldehyde gas under the conditions of the Falck-Hillarp method, forming fluorescent condensates The fluorescence yield was markedly improved (20 --30 times) if the formaldehyde condensation reaction was carried out in the presence of ozone Microspectrofluorometric analysis revealed that the excitation and emission maxima of the tryptophanyl-peptide fluorophores were very similar to those of the tryptamine fluorophore The properties of the tryptophanyl--peptide fluorophores differed from those of the tryptamine fluorophore in one important respect treatment with hydrochloric acid vapor during or after the formaldehyde condensation markedly enhanced the fluorescence intensity of tryptamine but not that of the tryptophanyl-peptides Thus, the combined formaldehyde--ozone treatment offers possibilities for the histochemical detection of N-terminal tryptophan-peptides The combined formaldehyde-HCl treatment can be used to distinguish tryptophanyl--peptides from tryptamine

J Hyttel[x)] and A Jergensen (The Research Laboratories H Lundbeck & Co A/S Ottiliavej 7-9 Copenhagen Denmark)
INHIBITION OF 5-HYDROXYTRYPTAMINE UPTAKE BY THYMOLEPTIC DRUGS MEASURED IN VIVO AND IN VITRO EFFECT OF CHLORSUBSTITUTION AND DEMETHYLATION

An in vivo and an in vitro method for measuring inhibition of 5 HT uptake by thymoleptic drugs have been compared

In the in vivo test was measured the ability of thymoleptics to inhibit the displacement of 5 HT from rat brain by 4-methyl-α-ethyl meta-tyramine (H 75/12) which is transported into the terminals of 5 HT neurons by the amine pump In the in vitro test was directly tested the inhibition of ^{14}C-5-HT uptake in rabbit blood platelets

A good correlation has been shown for the results from the two methods Drugs as amitriptyline for which the two methods are diverging showed pharmacological effects, not correlated to the thymoleptic effects influencing the results of the in vivo test

The effect of chlorsubstitution and demethylation of imipramine amitriptyline and a bicyclic thymoleptic was examined. The results show that chlorsubstitution enhances while demethylation reduces the inhibiting properties of the compounds

H Jõhannsson[x)] and E Nilsson (Farmakologiska Institutionen, Sölvegatan 10, Lund Sweden)
INFLUENCE OF ACID BASE CHANGES ON EXCITATION CONTRACTION COUPLING IN MYOCARDIUM

Isometric force and the intracellular action potential (AP) were measured in rabbit paipllary muscles at contraction rates of 12, 60 and 120 beats per min in solutions buffered with 10 mM L histi dine (pH 6 7 7 0

pronounced reduc

duration in the 47 mM HCO_3 solution being only 1/3 of that in the solutions without HCO_3 This indicates that contraction rate is of major importance in determining the myocardial inotropic response to changes in acid base parameters

J. Holm and C. Broen-Christensen
University of Copenhagen, Denmark)
INHIBITORY EFFECT OF CARBACHOL ON ... C_{10}
UPTAKE BY RAT KIDNEY CORTEX SLICES.

The quaternary ammonium compounds C_{10} and carbachol share a common transport mechanism in mouse kidney slices (Holm,J. : Acta physiol.scand. 1970, 80, 15A-16A). Rat kidney cortex slices accumulate C_{10} (McIsaac, R.J.: J.Pharmacol.1969,168, 6-12), and we have therefore found it of interest to perform the present study. Slices were incubated in Krebs-Ringer bicarbonate medium (pH 7.4,37°, aeration:95 % O_2 and 5 % CO_2) containing 14-C-C_{10} (2 µM) with or without carbachol, and slice-to-medium concentration ratio (S/M ratio) was measured. S/M ratio (1 hr) was increased ($P<0.001$) from 4.2 ± 0.3 to 7.5 ± 0.5 (mean ± S.E.M., N = 6) in the presence of 3 mM carbachol, whereas 50 mM carbachol reduced ($P<0.001$) S/M ratio from 5.0 ± 0.1 to 3.8 ± 0.2 (N = 8). Initial S/M ratio (5 min) was increased ($P<0.001$) from 0.95 ± 0.04 to 1.22 ± 0.03 (N = 9), when slices were preloaded (1½ hr) with 3 mM carbachol (in the absence of C_{10}) before transfer to a carbachol-free medium containing C_{10}. This suggests that stimulation is related to the presence of carbachol in the slices. No efflux (½ hr) occurred from slices preloaded (1 hr) with C_{10}, which means that stimulation cannot be attributed to inhibition of C_{10} efflux by internal carbachol. The stimulation phenomenon is interpreted as substrate-facilitated carrier transport, which should mean that carbachol efflux accelerates C_{10} influx.

H.HÜBERS*, E.HÜBERS, W.FORTH, G.LEOPOLD, W.RUMMEL (Inst.f. Pharmakol. & Toxikol. Homburg/Saar W.-Germany)
BINDING OF IRON AND OTHER METALS IN BRUSH BORDERS OF JEJUNUM AND ILEUM OF THE RAT IN VITRO.

The first step in the absorption of heavy metals in the gut is the contact with the brush borders (bb). Therefore, it was interesting to get informations about the binding properties of bb for heavy metals (Fe, Hg, Cu, Co, Zn, Mn; labelled by radioactive isotopes) in different gut segments. The bb were prepared using EDTA (MILLER, D. and CRANE, R.K.: Biochem.Biophys.Acta 52, 293 (1961)). The incubation medium was isotonic KCl (pH 7.4). Binding of metals was measured in dependence of temperature and concentration. With the exeption of ^{59}Fe no statistically significant difference was observed between the amount of metals bound to the bb of jejunum and ileum. The amount of ^{59}Fe taken up by the jejunal bb at 37°C increases proportionally to the amount presented. ^{59}Fe taken up by jejunal bb (0°C) could not be removed by subsequent washings. This indicates a rather stable binding of Fe by the acceptor sites. Binding of ^{59}Fe by ileal bb is labile. ^{60}Co can be removed of both jejunal and ileal bb easily.

P. Juul[x] and O.
Copenhagen Den.
SYMPATHETIC GANG...

Guanethidine was d
superior cervical g
of administration o
guanethidine monosulphate for 1 to 40 days The followi g
calculated as guanethidine base Guanethidine cumulated in the
sympathetic ganglia maximal amounts being determined following
administration for 2 3 weeks After 14 days administration of 20 mg/kg
an average of 27 ng/ganglion was found after 60 mg/kg an amount of
56 ng/g ... being 600 700µg Further
increas
of guan
hours The major pa...
since simultaneous administration of desmethylimipramine (25 mg/kg)
lowered the ganglionic ... to one f fth Thus the
concentration of guane
only a minor part of th
than the concentration
following prolonged administration was not lowered by
reserpinization The high concentrations of guanethidine in the
sympathetic ganglion cells are considered responsible for the
morphological alterations following prolonged administration of doses
above 10 mg/kg previously described by Jensen Holm J & P Juul
(Acta pharmacol. et toxicol 1970 28, 283 298)

W Kobinger[x] and A Walland (Arzneimittelforschung G m b H , Laskegasse 5-11, A 1120 Wien Austria) ENHANCEMENT OF VAGAL REFLEX BRADYCARDIA BY CLONIDINE PERIPHERAL OR CENTRAL NERVOUS EFFECT?

It was investigated whether the reflex vagal bradycardia, triggered by bloodpressure raising agents (BPA Noradrenaline 0,1 µg/kg, Angiotensin 0,1 µg/kg), is enhanced by Clonidine (St 155, Catapres) by means of its peripheral vasoconstrictor activity (Hoefke & Kobinger Arzneim -Forsch 1966 16, 1038-1050) or by a central action (vagal bradycardia see R D Robson, H R Kaplan & S Laforce J Pharmacol Exp Ther 1969, 169, 120-131) In dogs (Pentobarbital 30 mg/kg) after β-receptor inhibition with KÖ 592 (5 mg/kg i v 1-(Isopropylamino)-3-(m toloxy)-2-propanol) BPS were injected before and after St 155 and the heart rate was registered continously St 155 1 µg/kg injected into the cisterna cerebellomedullaris distinctly increased the reflex bradycardia The same dose i v had no effect It is considered that St 155 enhances vagal reflex bradycardia by a direct action in the CNS, as the drug exerts hypotension and bradycardia in vagotomized animals by a decrease in central nervous sympathetic activity (W Kobinger Naunyn-Schmiedebergs Arch Pharmak 1967, 258, 48-58 H Schmitt, Mme H Schmitt J R Boissier, J J Giudicelli & J Fichelle European J Pharmacol 1968, 2, 340-346)

H.G. Jonen, R. Kahl and G.F. Kahl[x] (Pharmakologisches Institut der Universität, Abteilung für Toxikologie, D 65 Mainz, Obere Zahlbacher Str. 67, Germany):
DEMONSTRATION OF "CYTOCHROME P-446" IN RAT LIVER MICROSOMES BY VARIOUS PYRIDINE DERIVATIVES.

Metyrapone, 2-methyl-1,2-bis(3-pyridyl)-1-propanone, has recently been shown to produce an absorption peak at 446 nm in reduced rat liver microsomes indicating the presence of a "cytochrome P-446" (Hildebrandt et al., Biochem. biophys. Res. Commun. 1969, 37, 477-485). In the present work it was demonstrated that during successive addition of small amounts of carbon monoxide to reduced microsomes from phenobarbital pretreated rats in the presence of metyrapone the absorption peak successively increased and shifted to 450 nm.

A number of other pyridine derivatives was also found to exhibit an absorption peak at 446 nm. The activity decreased in the following order: reduced metyrapone, 1-(2-pyridyl)-2-(4-pyridyl)-ethylene, 3-(3-pyridyl)-propanol, pyridine, 1-(2-pyridyl)-2-(3-pyridyl)-ethylene. The spectral changes caused by 10^{-3}M 1-(2-pyridyl)-2-(4-pyridyl)-ethylene and by 10^{-2}M 3-(3-pyridyl)-propanol were comparable to those observed with 10^{-4}M metyrapone. All substances which were able to produce an absorption peak at 446 nm were also shown to exhibit pyridine hemochromogen peaks with hemin. In experiments on the inhibitory action of 3-(3-pyridyl)-propanol on p-nitroanisole demethylation this substance turned out to be a less potent inhibitor than metyrapone.

A. Jørgen[x] [illegible] Overø (Research Laboratories, H. [illegible] iliavej 7-9, 2500 Copenhagen): THE [illegible] OF A SERIES OF THYMOLEPTICS COMPARED TO THEIR LIPID SOLUBILITY AND BINDING TO PLASMA PROTEINS.

The distribution pattern of 4 pairs (tert./sec. amines) of thymoleptics has been studied in rats, especially with reference to brain concentration. The four pairs are the tricyclic antidepressants, Amitriptyline/Nortriptyline and Melitracen/Litracen and two pairs of bicyclic compounds all labelled with carbon-14. The distribution pattern was determined by measuring total radioactivity and the radioactivity in the organs was identified by thin layer chromatography. The tricyclic compounds showed generally a relatively higher brain concentration and a lower concentration in lungs and liver as compared to the bicyclic compounds. Only minor differences were seen between the distribution of the tertiary amines and their corresponding secondary amines. The chromatographic analysis showed, that after administration (i.v.) of a tertiary amine, the brain contained almost exclusively unchanged drug, while the brain after administration of a secondary amine also contained metabolites especially the N-demethylated compound (primary amine). The relative lipid solubility and binding to plasma proteins were determined, but no obvious correlation was found between these parameters and the distribution pattern of the compounds.

P. Juul[x)] and O. Sand (D
Copenhagen, Denmark); (
SYMPATHETIC GANGLIA ...

administration for 2-3 weeks After 14 days administration of 20 mg/kg

only a minor part of the ganglionic volume was several times higher than the concentrations in non-sympathetic tissues The uptake following prolonged administration was not lowered by acute or chronic

W.Kobinger[x)] and ... G.m.b.H.,
Laskegasse 5-11,
REFLEX BRADYCARD... ENT OF VAGAL ... ENTRAL NERVOUS EFFECT?

It was investigated whether the reflex vagal bradycardia, triggered by bloodpressure raising agents (BPA. Noradrenaline 0,1 µg/kg, Angiotensin 0,1 µg/kg), is enhanced by Clonidine (St 155, Catapres) by means of its peripheral vasoconstrictor activity (Hoefke & Kobinger: Arzneim.-Forsch. 1966, 16, 1038-1050) or by a central action (vagal bradycardia see R.D.Robson, H.R.Kaplan & S.Laforce: J.Pharmacol. Exp.Ther. 1969, 169, 120-131). *In dogs* (Pentobarbital 30 mg/kg) after ß-receptor inhibition with KÖ 592 (5 mg/kg i.v. 1-(Isopropylamino)-3-(m-toloxy)-2-propanol) BPS were injected before and after St 155 and the heart rate was registered continously. St 155 1 µg/kg injected into the cisterna cerebellomedullaris distinctly increased the reflex bradycardia. The same dose i.v. had no effect. It is considered that St 155 *enhances vagal reflex bradycardia by a direct action in the CNS*, as the drug exerts hypotension and bradycardia *in vagotomized animals by a decrease in central* nervous sympathetic activity (W.Kobinger: Naunyn-Schmiedebergs Arch. Pharmak. 1967, 258, 48-58; H.Schmitt, Mme H. Schmitt, J.R.Boissier, J.J.Giudicelli & J.Fichelle: European J.Pharmacol. 1968, 2, 340-346).

H.G. Jonen, R. Kahl and G.F. Kahl[x] (Pharmakologisches Institut der Universität,
:, Obere Zahlbacher Str. 67, Germany):
ME P-446" IN RAT LIVER MICROSOMES

Metyrapone, 2-methyl-1,2-bis(3-pyridyl)-1-propanone, has recently been shown to produce an absorption peak at 446 nm in reduced rat liver microsomes indicating the presence of a "cytochrome P-446" (Hildebrandt et al., Biochem. biophys. Res. Commun. 1969, 37, 477-485). In the present work it was demonstrated that during successive addition of small amounts of carbon monoxide to reduced microsomes from phenobarbital pretreated rats in the presence of metyrapone the absorption peak successively increased and shifted to 450 nm.

A number of other pyridine derivatives was also found to exhibit an absorption peak at 446 nm. The activity decreased in the following order: reduced metyrapone, 1-(2-pyridyl)-2-(4-pyridyl)-ethylene, 3-(3-pyridyl)-propanol, pyridine, 1-(2-pyridyl)-2-(3-pyridyl)-ethylene. The spectral changes caused by 10^{-3}M 1-(2-pyridyl)-2-(4-pyridyl)-ethylene and by 10^{-2}M 3-(3-pyridyl)-propanol were comparable to those observed with 10^{-4}M metyrapone. All substances which were able to produce an absorption peak at 446 nm were also shown to exhibit pyridine hemochromogen peaks with hemin. In experiments on the inhibitory action of 3-(3-pyridyl)-propanol on p-nitroanisole demethylation this substance turned out to be a less potent inhibitor than metyrapone.

A. Jørgensen[x] and K. Fredricson Overø (Research Laboratories, H. Lundbeck & Co. A/S, Ottiliavej 7-9, 2500 Copenhagen): THE DISTRIBUTION PATTERN OF A SERIES OF THYMOLEPTICS COMPARED TO THEIR LIPID SOLUBILITY AND BINDING TO PLASMA PROTEINS.

The distribution pattern of 4 pairs (tert./sec. amines) of thymoleptics has been studied in rats, especially with reference to brain concentration. The four pairs are the tricyclic antidepressants, Amitriptyline/Nortriptyline and Melitracen/Litracen and two pairs of bicyclic compounds all labelled with carbon-14. The distribution pattern was determined by measuring total radioactivity and the radioactivity in the organs was identified by thin layer chromatography. The tricyclic compounds showed generally a relatively higher brain concentration and a lower concentration in lungs and liver as compared to the bicyclic compounds. Only minor differences were seen between the distribution of the tertiary amines and their corresponding secondary amines. The chromatographic analysis showed, that after administration (i.v.) of a tertiary amine, the brain contained almost exclusively unchanged drug, while the brain after administration of a secondary amine also contained metabolites especially the N-demethylated compound (primary amine). The relative lipid solubility and binding to plasma proteins were determined, but no obvious correlation was found between these parameters and the distribution pattern of the compounds.

KRISTENSEN, MARIE (Department of Pharmacology, University of Copenhagen, Juliane Maries Vej 20 DK 2100 Copenhagen Ø, Denmark) SECRETION OF GASTRIC HYDROCHLORIC ACID AND BUFFER SUBSTANCES BEFORE AND AFTER GLYCOPYRROLATE

In 12 patients with duodenal ulcer, gastric juice was aspirated one hour before and four hours after administration of the anticholinergic glycopyrrolate The pH, titratable acidity and the content of mucus were determined by a potentiometric procedure From the results it was possible to decide whether or not hydrochloric acid was secreted in excess of buffer substances

During the four hours following glycopyrrolate administration titratable acidity, expressed in percentages, was found to be 30 (range 16-53), 9 (0-41), 9 (0-39) and 19 (0-39), and the secretion of buffer substances 45 (range 20-83), 18 (7-58), 19 (5-62) and 26 (15-63)

During the hour before administration of glycopyrrolate the pH was below 4 5 in all samples allowing peptic activity, and in 10 out of 12 patients hydrochloric acid was secreted in excess of buffer substances

Although glycopyrrolate did not raise the pH of the samples above 4 5 in more than 3 of the 12 patients, it caused the buffer substances to be secreted in excess of hydrochloric acid in 8 out of the 12 patients

R Krowke G Bochert, P Mewes (Pharmakologisches Institut der FU Berlin Abt "Embryonal Pharmakologie", 1 Berlin 33 Thielallee 69/73, Germany)
EFFECTS OF SOME METABOLIC INHIBITORS ON NUCLEIC ACID SYNTHESIS IN EARLY STAGES OF MAMMALIAN EMBRYONIC DEVELOPMENT

Teratological studies hitherto mostly performed with morphological methods have to be supplemented by biochemical studies A procedure is presented which allows both a screening of main metabolic pathways and a closer analysis of the localisation of a metabolic defect within the nucleic acid metabolism of mammalian embryos (Krowke R et al , Naunyn Schmiedebergs Arch Pharmak 268, 229 234 1971 Krowke R et al , to be published in Naunyn Schmiedebergs Arch Pharmak) For this purpose ^{14}C-glucose and ^{32}P are given intravenously to pregnant experimental animals As an example the effect of cytosinarabinoside (ara-C) and hydroxyurea on the incorporation of the precursors into the ribose and base moieties of RNA and DNA is described in this paper With a number of antimetabolites tested the method developed has proven to be more sensitive in revealing embryotoxic effects than the usual morphological methods

Incorporation of ^{14}C-glucose fragments and ^{32}P-phosphate (3h) into different cell components of 12 day-old rat embryos after application of cytosinarabinoside and hydroxyurea (5h) in vivo expressed as % of controls

	mg/kg		RNA	DNA	Prot
Cyt - arab	10	^{14}C	134	102	132
		^{32}P	103	88	100
	30	^{14}C	125	74	147
		^{32}P	92	48	100
	100	^{14}C	126	53	143
		^{32}P	92	30	100

	mg/kg	RNA	DNA	Prot
Hydr - urea	250	97	77	123
		77	84	117
	500	65	42	117
		62	20	116
	750	39	30	98
		39	12	96

J. Kolberg[x)], K Helgeland and J. Jonsen (Department of Microbiology, Dental Faculty, University of Oslo, Norway)
ACCUMULATION OF LIPIDS IN CULTURED ANIMAL CELLS UPON INCUBATION WITH 2,4-DICHLOROPHENOXYACETIC ACID.

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) inhibits the growth of mouse fibroblasts (Earle's L 929 cells) in monolayer cultures (Kolberg, J., K. Helgeland, J. Jonsen, & O. Tjeltveit Acta Pharm. et Toxicol. 1971, 29,81-86). The inhibition is accompanied by a transient accumulation of lipid particles in the cytoplasm. Cells exposed to 500 µg of 2,4-D per ml for 24 hrs were found to contain 0.602 mg lipids (triglycerides, cholesterol and phospholipids) per mg of cell protein, as compared to 0.241 mg for the control cells. Triglycerides accounted for 72 per cent of this increase. Double-labelling experiments were performed with ^{3}H-palmitate and ^{14}C-acetate. The incorporation of ^{3}H-palmitate into the cell lipids increased 96 per cent upon exposure to 2,4-D, whereas the specific activity of the triglycerides from 2,4-D treated cells and control cells remained similar. The specific activity of ^{14}C-acetate in triglycerides from cells exposed to 2,4-D was only 27 per cent of that in the control cells. These findings indicate that the formation of lipid particles was due to an increased uptake of fatty acids, and was not related to an increased endogenous synthesis.

. .. . A.Ladous and D.Ettehadieh
versität, 78 Freiburg, BRD)
/A RHIZOME (PIPER METHYST.).

To clarify the reason of the sedative action of the polynesian kava drink we quantitatively studied the effect of the isolated pyrone constituents kavain, methysticin, dihydromethysticin and yangonin on the skeletal muscle tone of unanesthetized rabbits, electromyographically measured from the calf muscle during periods of a constant flexion of a paw (Teschendorf, H.J., R.Kretzschmar & A.Ladous Arch.Pharmak. 1970, 266, 467). Further we studied their action on the electrical activity of cortical and subcortical brain structures of rabbits (Monnier-technic). All the kavapyrones showed a strong centrally caused muscle relaxing activity. Yangonin proved to be the most potent kavapyrone. 5-10 mg/kg i.v. nearly completely depressed the EMG impulses. 2-3 times larger doses of the other pyrones showed the same effect. With relaxing doses of the pyrones high voltage synchronized waves developed in the cortical EEG with only slight diminution in frequency. They further depressed without completely abolishing the electrically and acoustically induced arousal reaction and significantly shortened the duration of the after-discharges following the electrical stimulation of the dorsal hippocampus. The same doses only slightly increased the threshold of the recruiting response. The results indicate, that the sedativ effect of the drug in man probably is the consequence of both the depression of muscle tone and the depression of the cortical activation system and limbic areas by the pyrone compounds.

P. G. Lankisch[x] and W. Vogt (Dept. of Biochemical Pharmacology, Max-Planck-Institut f. exp. Medizin, D-3400 Gottingen, Germany): DEPENDENCE OF THE LYTIC ACTIVITY OF PHOSPHOLIPASE A ON THE TENSION OF THE RED CELL MEMBRANE.

Earlier results suggested that the direct lytic factor of cobra venom (DLF) and similarly acting peptides potentiate phospholipase A haemolysis by causing the red cells to swell. The increase in volume should lead to stretching of the membrane and give access of phospholipase A to membrane phospholipids which are normally hidden (Lankisch, P.G. & W. Vogt: Naunyn-Schmiedebergs Arch. exp.Pharmak., in print). This assumption is supported by the following new findings:

(1) Prymnesin, a proteo-lipid toxin of algae, which causes osmotic haemolysis, potentiates phospholipase A at low, sub-lytic concentrations.

(2) The enhanced lytic activity of phospholipase A in hypotonic solution is not due to the additive effects of mechanical and biochemical labilization of the membrane. Haemolysis is not enhanced when phospholipase A acts in normotonic solution and the cells are subsequently transferred to the hypotonic medium without enzyme.

(3) In hypotonic solution phospholipase A cleaves more cell phospholipids than in normotonic medium.

(4) Cells from patients with hereditary spherocytosis are more susceptible to phospholipase A haemolysis than normal human red cells.

C. Larsson[x] and E. Änggård (Department of Pharmacology, Karolinska Institutet, S 104 01 Stockholm 60, Sweden)
ON THE FORMATION OF PROSTAGLANDIN E_2 IN THE RABBIT RENAL PAPILLA.

The present study was undertaken to provide evidence as to the subcellular localization of prostaglandins (PG), PG precursor acids, PG synthetase and 15-hydroxy PG dehydrogenase (PGDH) in the rabbit renal papilla. Most of the smooth muscle material (rat fundus strip) was determined to be PGE_2 and was present in the supernatant. The concentration of PGE_2 was 14-39 $\mu g/g$ of which about 50 % was formed during preparation of the fractions. Arachidonic acid was the major PG precursor acid (290 ± 63 $\mu g/g$) and occurred most abundantly in microsomes, mitochondria and supernatant. PG synthetase was present mostly in the microsomes. No PGDH was present in the papilla but occurred in high concentrations in the medulla and cortex. The results show that high amounts of PGE_2 are formed in microsomes probably from locally available arachidonate. After its formation PGE_2 is released into the cytosol rather than being concentrated within any subcellular compartments and may leave the papilla without undergoing inactivation by metabolism.

H Kunze[x], G Rehbock and W Vogt (Dept of Biochem Pharmacology, Max-Planck-Institut f exp Medizin, Hermann-Rein-Str 3, D-3400 Gottingen, Germany) ABSORPTION OF o-, m- AND p-HYDROXYBENZOIC ACID FROM THE RAT JEJUNUM

Absorption of ^{14}C-hydroxybenzoic acids was studied in situ in tied jejunal loops of rats, anaesthetized with urethane The rate of absorption was determined as described earlier for ^{14}C-neostigmine (Kunze, H , K Arens & W Vogt: Naunyn-Schmiedebergs Arch Pharmak 1970, 266, 385) For all isomers nearly linear correlations exist between the quantities absorbed and the concentrations given, and between log of the percentage remaining in the loop and time, indicating that these substances are probably absorbed by diffusion alone The rate of absorption decreases in the order o- ≫ p- > m-hydroxybenzoic acid The extremely high absorption rate of the o-isomer obviously results from penetration of the non-ionic as well as ionic form This is suggested also by the finding that EDTA which facilitates the penetration of hydrophilic substances increased the absorption of o-, although it decreased that of m- and p-hydroxybenzoic acid The hypothesis is advanced that o-hydroxybenzoic acid, because of its chelating property, binds metal ions at the surface or within the cell in this way it directly or indirectly (by inhibiting cell metabolism) leads to an increased permeability for water soluble substances, this would explain the high absorption rate of o-hydroxybenzoate ions

K Kuschinsky (Max-Planck-Institut für experimentelle Medizin Dept of Biochemical Pharmacology, D-3400 Göttingen, Hermann-Rein-Str 3, Bundesrep Deutschland): EFFECT OF MORPHINE ON PROTEIN SYNTHESIS IN SYNAPTOSOMES AND MITOCHONDRIA OF MOUSE BRAIN

The effect of morphine on incorporation of ^{14}C-leucine into proteins of synaptosomes and mitochondria of mouse brain was studied in vivo The subcellular fractions were prepared according to Gray and Whittaker (J Anat 1962, 96 79-88) The animals were injected with 100 mg/kg morphine i p or with saline respectively, and 15 min later with 100 µCi/kg L-leucine 1-^{14}C 4 hours after this injection, the animals were killed and the subcellular fractions of the brains prepared and the proteins precipitated Morphine significantly decreased the uptake of ^{14}C-leucine into the synaptosomes by about 20% It did not affect the uptake into the mitochondria, however The effect was dose-dependent and could be prevented by naloxone 200 mg/kg barbital given instead of morphine depressed the incorporation of ^{14}C-leucine into synaptosomes and mitochondria both by about 15% Thus the inhibitory effects of both substances differ in their patterns The depressing effect of morphine on uptake of ^{14}C-leucine into the synaptosomes in vivo is probably due to inhibition of axoplasmic transport of proteins, and is an effect of higher specificity than that of barbital

S. Lindahl*), T. Wåhlstrand and P. Wistrand (Department of Medical Pharmacology, University of Uppsala, Uppsala, Sweden) KINETICS OF HUMAN KIDNEY CARBONIC ANHYDRASE.

Carbonic anhydrase was prepared from donor kidneys perfused with Perfadex. The perfused kidney was virtually free from contaminating erythrocytes since no (< 1.5 µM) erythrocyte carbonic anhydrase isozyme HCA B and only traces of hemoglobin (< 0.1 mg/g) were found in the kidney homogenate. The enzyme was isolated from the supernatant of the centrifuged (105.000 g for 60 min.) homogenate (Falkbring, S.O., Nyman, P.O., Wåhlstrand, T., & Wistrand, P. see abstract of this meeting) by chromatography. The enzyme activity of the homogenate was tested kinetically using the changing pH-method of Philpot & Philpot (Biochem J. 1936, 30, 2191). The purified enzyme was tested in a stopped-flow apparatus. CO_2, HCO_3^- and p-nitrophenyl acetate were used as substrates; sulfanilamide and thiazides were used as inhibitors. The carbonic anhydrase of human kidney was found to behave kinetically like the high activity carbonic anhydrase isozyme HCA C of the human erythrocytes.

W Lorenz x), A Doenicke K Messmer and J H Reimann
Abteilung für experimentelle Chirurgie und pathologische Biochemie, Chirurgische Univ Klinik Marburg a d Lahn, Germany, Robert Koch-Straße 8 HISTAMINE RELEASE IN MAN AND DOG BY PLASMA SUBSTITUTES

Histamine release in man after rapid infusion of plasma substitutes on gelatine base according to the method of Messmer et al , Arch Pharmakol. 1970, 267, 433-445 could be shown by measuring an increase of plasma histamine concentration by 350 % and of gastric acid secretion Histamine was determined by a new fluorometric method (Lorenz et al , Z anal Chem. 1970, 252, 94-98) the specificity of which was proved by enzymatic inactivation of the substance isolated from the plasma in the presence of 120-fold purified histamine methyltransferase from pig antral mucosa and 165 fold purified diamine oxidase from pig kidney The mean value of plasma histamine concentration in 54 normal human beings was found to be 0,69 ng/ml Ringer's solution and dextran-60 showed no histamine release.
Also in dogs, only plasma substitutes on gelatine base released histamine The determination of the amine in blood from different regions of circulation and in various tissues showed, that histamine was released predominantly in the skin, but not in liver, lung and gastrointestinal tissues.

J. Buus Lassen '
Copenhagen, Den! .. , A/S Ferrosan
RATS AFTER INHI! ... SYNTHESIS, STORAGE AND F AMANTADINE IN
RECEPTORINTERACTION.

The behavioural effect of amantadine has been studied in rats. After 25-50 mg/kg s.c. increased locomotion, sniffing, rearing, head twitch and spells of rapid grooming with forelegs was observed. The same symptoms and a weak tremor was registered after 100 mg/kg. The motility was measured using an Animex activity meter (Svensson, T.H. & G. Thieme, Psychopharmacologia 1969, 14, 157-163). The effect of amantadine 50-100 mg on locomotor activity was investigated after s.c. administration of various drugs interfering with monoamine function. Aceperone 10 mg/kg, spiramide 0.1 mg/kg and perphenazine 0.2 mg inhibited the amantadine-induced hyperactivity. Reserpine 7.5 mg/kg and α-methyl-p-tyrosine (H44/68) 250 mg/kg exerted partial inhibition. Reserpine + H44/68 or tetrabenazine potentiated the effect of amantadine. The amantadine-induced hyperactivity in rats pretreated with reserpine + H44/68 or tetrabenazine could not be blocked by scopolamine, suggesting that this behaviour is not due to a central cholinergic predominance but to a direct stimulation of adrenergic receptors. The stimulant effect of amantadine in rats does not show clear resemblance to the effect of known stimulant drugs. No stereotyped licking or gnawing was found even after high doses of amantadine.

J. Buus Lassen,[x)] J.A. Christensen, J. Lund and R.F. Squires (Research Laboratories, A/S Ferrosan, Copenhagen, Denmark):
PHARMACOLOGICAL AND BIOCHEMICAL STUDIES ON 2-AMINO-4-PHENYLSULFONYLBENZENESULFONAMIDE (NSD3004), A NEW SULFONAMIDE WITH ANTICONVULSANT AND CARBONIC ANHYDRASE INHIBITORY PROPERTIES.

After oral administration to mice NSD3004 protected against maximal electroshock seizures in a dosage of 10-20 mg/kg. Phenobarbital and diphenylhydantoin had about the same activity. The effect of NSD3004 was correlated with the plasma concentration of the substance. No significant tolerance to the anticonvulsant effect of NSD3004 was found after daily oral administration for 14 days. Using in vitro and in vivo techniques NSD3004 was found to possess carbonic anhydrase inhibition. The most striking difference between NSD3004 and the carbonic anhydrase inhibitor acetazolamide was the ratio of anticonvulsant effect to diuretic activity. NSD3004 exerted a strong anticonvulsant and weak diuretic effect while the opposite relation between the two effects was found with acetazolamide. When NSD3004 and acetazolamide were administered orally in the same doses, NSD3004 showed a stronger carbonic anhydrase inhibition in brain and a weaker inhibition in kidney than acetazolamide. However, the inhibition was underestimated by the method used since the organ homogenates were considerably diluted and the two inhibitors are known to be reversible. Inhibition of carbonic anhydrase in brain may be related to the anticonvulsant effect of NSD3004 but conclusive evidence is lacking.

P Lundborg[x] and C Kellogg (Department of pharmacology, Fack, 400 33 Göteborg 33, Sweden) UTILISATION OF ^{3}H-DOPA IN THE BRAIN DURING PRE- AND POSTNATAL DEVELOPMENT

The formation of tritiated noradrenaline (^{3}H-NA) and dopamine (^{3}H-DA) and their O-methylated metabolites was analyzed in the brains of rats during pre- and post- [illegible] ection of tritiated 3,4-dihydroxyphenyl- [illegible]

weeks and received a subcutaneous injection [illegible] p- ared between animals pretreated with an inhibitor of peripheral l-aminoacid decarboxylase (MK-486) and control animals given only saline Pretreatment of the mother with MK-486 produced an identical increase in the level of ^{3}H-DA in the brain of both the mother and foetuses However, the level of ^{3}H-NA in the foetal brain increased to only 60% of the increase observed in the mothers' brains In the young animals, an increase in the concentration of ^{3}H-NA and ^{3}H-DA in the brain was observed from newborns to four days followed by a decrease at three weeks The level of ^{3}H-DA reached after MK-486 pretreatment was almost the same in all three age groups The level of ^{3}H-NA reached increased with age MK-486 pretreatment decreased the level of the O-methylated metabolites in the newborn and four-day old rats but increased these levels in the three week old animals

These results indicated that the l-aminoacid decarboxylase is fully developed in the brains of the fullterm foetuses However, peripheral decarboxylase activity appears to be low at birth and develops with age The ontogenic development of this enzyme markedly influences the level of the tritiated amines produced in the brain following the peripheral injection of the tritiated precursor

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ACTIVITY

Atropine enhances the acetylcholine (ACh) release from exposed cortex (Mitchell, J P J Physiol 1963, 165, 98-118) and in large doses increases the locomotor activity in rats (Longo, V G.: Pharmacol. Rev. 1966, 18, 965-996) and induces the central anticholinergic syndrome (CAS) in dogs (Albanus, L Acta Pharmacol Toxicol 1970, 28, 305-326). Potent psychotomimetic glycolates (3-quinuclidinylbenzilate and 3-quinuclidinyl 3,3'-thenilate) also produces CAS in dogs. Therefore their effect on ACh release from exposed cortex of unanaesthetized rats and on locomotor activity was investigated after i v administration. It was found that they qualitatively resembled atropine in these respects and that their potencies were roughly correlated to their threshold doses to elicit CAS. The onset of CAS and of some peripheral anticholinergic effects has been reported to be slower after these glycolates than after atropine (Albanus, L Acta Pharmacol Toxicol 1970, 28, 305-326). After glycolate administration there was also a gradual increase of ACh release with a maximal effect after about 90 minutes while the atropine induced release was maximal during the first 30 minutes Also after local cortical application this difference in time course was evident. The ACh releasing potency of these glycolates thus seems correlated to their anticholinergic potency while the mechanism behind their slow onset is unexplained.

W.Lorenz[x)], H.Troidl, G.Feifel, A.Huhnd, H.Barth and H.Rohde
Abteilung für experimentelle Chirurgie und pathologische Biochemie, Chirurgische Univ.-Klinik Marburg a.d.Lahn, Germany, Robert-Koch-Straße 8: INHIBITION OF GASTRIC HISTAMINE METHYLTRANSFERASE BY AMODIAQUINE IN VITRO AND IN VIVO.

Amodiaquine, an antimalarial drug, strongly inhibited histamine methyltransferase from gastric mucosa of pig and dog in vitro. Studies on 5 dogs with Heidenhain pouches showed that amodiaquine (2 mg/kg i.m.) increased gastric acid secretion induced by pentagastrin (6 μg/kg i.m.) and betazole (2 mg/kg i.m.) by 60 % on the average. The effect was dependent on the dose of amodiaquine (0,5-3,0 mg/kg i.m.) and pentagastrin (1-6 μg/kg i.m.); for lower doses of pentagastrin higher doses of amodiaquine were necessary to evoke a maximal increase of secretion. The antimalarial drug did not influence the pancreatic secretion of dogs elicited by secretin. The action on salivary glands of dogs varied from animal to animal. Partly an increase of pilocarpine (40 μg/kg i.a.) induced secretion was observed, partly an inhibition occured. The increase of gastric acid secretion in dogs after treatment with amodiaquine can be interpreted as an inhibition of gastric histamine methyltransferase in vivo. This would be an argument for the hypothesis that histamine is a physiological chemostimulator of gastric acid secretion in dogs.

J. Lund[x)], J. Buus Lassen and R.F. Squires (Research Laboratories, A/S Ferrosan, Copenhagen, Denmark): THE DISTRIBUTION OF 2-AMINO-4-PHENYLSULFONYLBENZENESULFONAMIDE (NSD 3004) IN BLOOD AND ITS BINDING TO BLOOD COMPONENTS.

The biological half-life of NSD 3004 in blood shows a great variation in different species. In man the half-life in blood is up to 6 months, in dog 30 days, in pig 17 days, and in rabbit about 2 days. In a distribution study (human blood) no substance could be detected in plasma at blood concentrations below 120-150 μM. This made it possible to determine the receptor (drug) concentration ($R_{tot.}$) in red blood cells in a simple distribution study. The dissociation constant of the drug-receptor complex ($K_{diss.}$) has been determined after suspending red blood cells in 0.9% NaCl. $K_{diss.}$ was found to be 10^{-7} - 10^{-8} M. Besides the large affinity to the red blood cells NSD 3004 is bound to the plasma proteins (90-100%). In the red blood cells NSD 3004 is bound to carbonic anhydrase (method of Armstrong, J.: J.Biol.Chem. 1966, 241, 5137-5149). The results show that NSD 3004 has a high affinity to the carbonic anhydrase in the red blood cells and that this may be the reason for the long half-life in human blood. The values of R_{tot}, and the fall in blood concentration in human, indicates that the substance is bound to both HCAB and HCAC with the highest affinity to HCAC.

R Lynen[x], H Kunze, and W Vogt (Dept of Biochem Pharmacology Max Planck-Institut f exp Medizin, Hermann-Rein Str 3, D-3400 Göttingen, Germany) EFFECT OF SALICYLIC ACID AND ITS ISOMERS ON THE JEJUNAL ABSORPTION OF ^{14}C-MANNITOL AND PHENOL RED IN THE RAT

The intestinal absorption of ^{14}C-mannitol in the rat jejunum was followed over a period of 120 min, using tied loop preparations The proportion absorbed determined as described earlier (Kunze, H , K Arens & W Vogt: Naunyn-Schmiedebergs Arch Pharmak 1970, 266, 385), was 6 8±2 8% In the presence of salicylic acid the absorption was increased to 20 5±8 0%; m- and p-hydroxybenzoic acid led to a slight enhancement of absorption of mannitol, 11 6±3 7% and 11 2±4 2%, respectively The absorption of phenol red (5mM) determined by the method of R R Levine (J Pharmacol exper Ther 1961, 131, 328-333) after 120 min was not altered by 50mM salicylic acid Only at a concentration of 100mM the phenol red absorption was increased from 3 5±1 3% to 6 1±2 0% However this effect was not specific, as 100 mM m-hydroxybenzoic acid acted in the same way The results demonstrate that salicylic acid specifically improves the absorption of mannitol and probably other hydrophilic substances The effect appears to be limited by the molecular size of the substances concerned Salicylic acid might produce this effect by chelation of metal ions at the absorptive cell surface (thus enlarging the pore radius) and/or within the cell (producing alterations of membrane permeability by metabolic disturbance) Further studies should help to determine the significance of each of the two mechanisms

E Marmo[x], A P Caputi, (Istituto di Farmacologia e Tossicologia , Università di Napoli, Italy) KARDIOVASKULÄRE WIRKUNGEN VON PRENYLAMIN UND EINIGEN DERIVATEN (HOE 782, HOE 976 und HOE 674)

Es wurden die Wirkungen von Prenylamin und drei seiner Derivate (HOE 982 /dl-1,1-m-hydroxy-phenyl-2-(γ ,γ -diphenyl-propylamine)-propanol)/, HOE 976 , /dl-1-(3',4'-dimethylphenyl)2-(diphenyl-propylamine)-propanol/, HOE 674, /dl-1-(3', 4 -dimethylphenyl)-2-(phenylpyridyl-propylamine)-propanol/ auf den Blutdruck der Katze und das EKG der Meerschweinchens untersucht Fur Prenylamin wurden nach Verabreichung angemessener Dosen hypotensive, bradycardisierende, anti-coronarospastische und antiarrhythmische Wirkungen festgestellt, der hypertensive Effekt von Noradrenalin wurde gesteigert, β-adrenolytische Wirkungen auf den Blutdruck der Katze wurden nicht festgestellt Die Derivate von Prenylamin (HOE 782, HOE 976 und HOE 674) wirkten, in angemessenen Dosen gegeben, hypotensiv, bradykardisierend, anticoronarospastisch, antiarrhythmisch und β-adrenolytisch Der hypertensive Effekt von Noradrenalin wurde verstärkt

Ingmar Lundquist (Department of Pharmacology, University of Lund, Lund Sweden): GLUCOSE UTILIZATION AND ADRENERGIC RECEPTORS IN MUSCLE AND LIVER TISSUE.

The significance of adrenergic metabolic receptors in muscle and liver utilization of glucose was studied in mice by α-adrenergic (phentolamine) and β-adrenergic (L-propranolol) blockade in vivo and in vitro. Incubation of muscle with glucose-U-^{14}C in the presence of L-propranolol or D-propranolol (control) yielded the following results: L-propranolol (L-prop) induced an increased $^{14}CO_2$-production and ^{14}C-incorporation intc glycogen. Total glycogen in L-prop treated muscle was increased. These effects were also seen when L-prop was given in vivo and the muscle subsequently incubated in vitro. Similar results were seen after incubation of muscle with L-prop in the presence of L-adrenaline (L-adr) and L-adr + insulin. α-adrenergic blockade with phentolamine had no effect. Incubation of liver showed that both α- and β-adrenergic blockade, respectively, increased total glycogen in the presence of L-adr and L-adr + insulin. Injection of liver glycogen depleting doses of insulin in vivo was counteracted by pretreatment with phentolamine + L-prop, whereas injection of either drug alone produced only partial antagonism.
The results suggest: 1) The occurrence of a β-receptor mediated effect on the glycogen regulating system in muscle, and also the existence of β-adrenergic mechanisms which influence the membrane transport of glucose. 2) The glycogen regulating system in the liver may be controlled by both α- and β-adrenergic receptors. The major effect of adrenaline on liver glycogen is exerted on glycogen synthesis.

H. Lüllmann+ and A. Ziegler (Department of Pharmacology, Hospitalstr. 4-6, 23 Kiel, W.Germany): THE KINETICS OF THE RELEASE OF ^{3}H-ATROPINE BY ISOLATED GUINEA PIG'S ATRIA.

In experiments with isolated electrically driven guinea pig's atria the time course of the wash-out of ^{3}H-atropine has been investigated. The very slow release of atropine from the tissue was not only determined by a low dissociation rate from the binding sites but also by re-adsorption of atropine molecules present in the extracellular space. - The release of atropine was not enhanced by even high concentrations of carbachol. However, the presence of pilocarpine, norarecoline or arecaidine-n-propylester, also muscarinic acting drugs, accelerated the release dose dependent. - The kinetics of the release - (and uptake-) curves of atropine by atrial tissue can be described comprehensively only if the following is assumed: The atropine molecule is bound to the muscarinic receptor and additionally to an adjacent filed via the hydroxymethylene-group. Under control conditions the over all dissociation rate constant determine the release. In the presence of agonists with low dissociation rate constants like pilocarpine, nor-arecoline and arecaidine-n-propyl-ester, atropine becomes detached from the muscarinic receptor, the dissociation rate constant of the additional binding becomes rate determining for the release of atropine. The dissociation rate constant of the additional binding is larger than the over all dissociation rate constant of atropine, but still noticeably smaller than that of the carbachol receptor binding thus permitting a short lasting interaction of the agonists with the receptor without a final displacement of atropine.

R. Lynen[x], H. Kunze, and W. Vogt (Dept. of Biochem. Pharmacology, Max-Planck-Institut f. exp. Medizin, Hermann-Rein-Str. 3, D-3400 Gottingen, Germany): EFFECT OF *SALICYLIC ACID* AND ITS ISOMERS ON THE JEJUNAL ABSORPTION OF *^{14}C-MANNITOL* AND PHENOL RED IN THE RAT.

The intestinal absorption of ^{14}C-mannitol in the rat jejunum was followed over a period of 120 min, using tied loop preparations. The proportion absorbed, determined as described earlier (Kunze, H., K. Arens & W. Vogt: Naunyn-Schmiedebergs Arch.Pharmak. 1970, 266, 385), was 6.8±2.8%. In the presence of salicylic acid the absorption was increased to 20.5±8.0%, m- and p-hydroxybenzoic acid led to a slight enhancement of absorption of mannitol, 11.6±3.7% and 11.2±4.2%,respectively. The absorption of phenol red (5mM) determined by the method of R.R.Levine (J.Pharmacol.exper.Ther. 1961, 131, 328-333) after 120 min was not altered by 50mM salicylic acid. Only at a concentration of 100mM the phenol red absorption was increased from 3.5±1.3% to 6.1±2.0%. However this effect was not specific, as 100 mM m-hydroxybenzoic acid acted in the same way. The results demonstrate that salicylic acid specifically improves the absorption of mannitol and probably other hydrophilic substances. The effect appears to be limited by the molecular size of the substances concerned. Salicylic acid might produce this effect by chelation of metal ions at the absorptive cell surface (thus enlarging the pore radius) and/or within the cell (producing alterations of membrane permeability by metabolic disturbance). Further studies should help to determine the significance of each of the two mechanisms.

E. Marmo[x], A.P. Caputi, (Istituto di Farmacologia e Tossicologia , Università di Napoli, Italy): KARDIOVASKULÄRE WIRKUNGEN VON PRENYLAMIN UND EINIGEN DERIVATEN (HOE 782, HOE 976 und HOE 674).

Es wurden die Wirkungen von Prenylamin und drei seiner Derivate (HOE 982, /dl-1,1-m-hydroxy-phenyl-2-(γ ,γ -diphenyl-propylamine)-propanol)/; HOE 976 , /dl-1-(3',4'-dimethylphenyl)2-(diphenyl-propylamine)-propanol/, HOE 674, /dl-1-(3',4'-dimethylphenyl)-2-(phenylpyridyl-propylamine)-propanol/ auf den Blutdruck der Katze und das EKG der Meerschweinchens untersucht. Für Prenylamin wurden nach Verabreichung angemessener Dosen hypotensive, bradycardisierende, *anti-coronarospastische* und antiarrhythmische Wirkungen festgestellt, der hypertensive Effekt von Noradrenalin wurde *gesteigert;* β-adrenolytische Wirkungen auf den Blutdruck der Katze wurden nicht festgestellt. Die Derivate von Prenylamin (*HOE 782, HOE* 976 und HOE 674) wirkten, in angemessenen Dosen *gegeben, hypotensiv*, bradykardisierend, anticoronarospastisch, *antiarrhythmisch* und β-adrenolytisch. Der hypertensive Effekt von Noradrenalin wurde verstärkt.

M.Meier (Biological Research Laboratories, Pharmaceutical Division of CIBA-GEIGY Limited, Basle, Switzerland): INOTROPIC ACTIVITY OF OUABAIN IN PAPILLARY MUSCLES (PM) OF CATS WITH RIGHT VENTRICULAR HYPERTROPHY.

In cats right ventricular hypertrophy was produced by pulmonary artery constriction (PAC) according to the method of Spann et al. (Proc.Soc. Exp.Biol.Med. 125, 522-524, 1967) or a sham-operation (SO) was performed. 7-183 days postoperatively the inotropic effects of 0.0625 or 1.0 µg/ml Ouabain were investigated in right ventricular PM contracting isometrically near the apex of the length-tension curve (stimulation frequency 12/min; Krebs-Henseleit solution; 30°C). The PM of cats with PAC showed marked depression of contractility: developed tension (DT) was 2.59±0.25 g/mm^2 and maximal rate of tension development (MRTD) 11.50±1.06 g/mm^2/sec (n=24). In PM of SO-cats DT was 4.85±0.29 g/mm^2 and MRTD 22.94±1.67 g/mm^2/sec (n=21). Resting tension was the same in both groups. 0.0625 µg/ml Ouabain increased DT in 12 PM of cats with PAC and in 10 PM of SO-cats by 0.93±0.13 and 1.19±0.11 g/mm^2 and MRTD by 4.33±0.66 and 7.36±1.51 g/mm^2/sec ($p<0.05$). 1 µg/ml Ouabain increased DT in 12 PM of cats with PAC and in 11 PM of SO-cats by 3.84±0.49 and 3.69±0.26 g/mm^2 and MRTD by 25.83±3.33 and 27.55±2.08 g/mm^2/sec. Thus, a threshold concentration of Ouabain produced a slight subnormal response in PM with reduced contractility, whereas the effect of a toxic concentration of Ouabain was not changed.

A. Melander[x], L.E. Ericson, R. Håkanson, Ch. Owman and F. Sundler (Department of Pharmacology, University of Lund, Lund, Sweden): SIGNIFICANCE OF THYROID MAST CELLS IN THYROID HORMONE SECRETION.

The relations of plasma TSH, amine-containing thyroid mast cells, and thyroid hormone secretion were studied in the rat by a combination of bio-assay, chemical, histochemical, and electron microscopical procedures.

TSH promoted a rapid and reversible depletion of 5-HT and histamine from intra- (but not from extra-) thyroidal mast cells, and a concomitant decrease in their metachromasia, whereas their electron-dense granules were neither extruded nor altered.

A single dose of 48/80 or 5-HT induced a release of thyroid hormone, as did TSH. The TSH-induced release of thyroid hormone was diminished when thyroid mast cells had been eliminated by chronic 48/80 treatment.

Thus, TSH induces a specific, non-degranulative release of 5-HT, histamine, and possibly heparin from thyroid mast cells. This process may participate in the secretion of thyroid hormone.

H. Meyer* and H.-H. Frey (Dept. of Pharmacol. and Toxicol., School of Vet.-Med., Free Univ., Berlin, Germany) SIGNIFICANCE OF CENTRAL BIOGENIC AMINES FOR THE ANTICONVULSANT EFFECT OF PHENOBARBITAL.

[illegible] level and turnover of the central biogenic [illegible]

the oral ED50 for the drug was 21 mg/kg [illegible] phenylalanine (300 mg/kg orally on 3 days before the test) raised the ED50 to 34 mg/kg, and low doses of cyproheptadine (0.1 - 0.2 mg/kg) acted in the same direction, whereas treatment with 100 mg/kg 5-HTP caused a decline to 15 mg/kg. Pretreatment with 100 mg/kg α-methyltyrosine 4 h before the test provoked a significant increase of the ED50 to 28 mg/kg and the same increase resulted from the use of phentolamine (1 mg/kg 20 min before electroshock). Propranolol, disulfiram and haloperidol were without influence on the anticonvulsant potency of phenobarbital. Small doses of chlorpromazine (0.1 - 0.5 mg/kg) proved to be synergistic with phenobarbital, doses of 1 and 2 mg/kg were without effect, and higher doses antagonized the effect of the anticonvulsant. p-Chloroamphetamine, though reducing the turnover of central 5-HT considerably, showed a definite synergism with phenobarbital which is considered to be independent of the effect of this drug on central monoamines. The results point to a significance of at least 5-HT and noradrenaline for seizure threshold and anticonvulsant drug action.

B. Meyerson* and L. Lindström (Department of Pharmacology, University of Uppsala, Sweden): SEXUAL MOTIVATION IN THE NEONATALLY ANDROGEN TREATED FEMALE RAT.

It is well established that psychosexual differentiation is influenced by hormone treatment during a critical neonatal period. In the female rat testosterone at 5 days of age disturbs the normal adult display of mating behaviour. Less is known about the effect on the urge to seek heterosexual contact, i.e. sexual motivation. In an "open field" the subjects are observed on an observation arena, a stimulus animal located in the center, the experimental animal free to move around. By a camera placed in the cage ceiling the location of the experimental animal in relation to the incentive animal was recorded. Another method, "increasing barrier", was designed to measure the willingness of the animal to pay a painful stimulus to reach a sexually active partner. The subjects have to pass via an electric grid from one cage to a second one which holds the incentive animal. The intensity of the grid is stepwise increased every second time the subject passes. The amount of barrier chock the animals are willing to bear is recorded.
Female Sprague-Dawley rats were given testosterone propionate (TP) 1 mg/kg s.c. (Neo-TP) or oilblank solution 0.05 ml s.c. (Neo-oil) at 5 days of age. Ovariectomy was performed when 90 days old. In the Neo-oil treated animals estradiol benzoate, 100 ug/kg s.c. induced an evident urge to seek contact with a vigorous male. This was a consistent finding in the two methods used. The neonatally testosterone treated females did not display any urge to seek contact with a vigorous male nor with a sexually active female. It is concluded that the adult heterosexual motivation was disturbed by the early androgen treatment.

B.J.Meyerson , E-B. Nordstrom and A.Ågmo (Department of Pharmacology, University of Uppsala, Sweden): SEXUAL BEHAVIOUR AND TESTOSTERONE IN THE FEMALE RAT.

The ability of testosterone propionate (TP) to activate sexual motivation (the urge to seek sexual contact) and mating behaviour (lordosis response on mounting by a male) was investigated in the female rat, ovariectomized as adult.
Sexual motivation was measured as the willingness of the animal to bear a painful stimulus (electric grid) to reach contact with a sexually active stimulus animal (increasing barrier method, Meyerson & Lindstrom Proc. 3rd Steroid Congr., Hamburg 1970). The experiments were performed one run a day for 16 consecutive days starting one day before hormone treatment. TP (0.5 - 1.0 mg/kg s.c.) significantly increased the number of grid-crossin s when the incentive animal was a male. The maximal response was seen 5 days after TP treatment. A similar effect was obtainel if the stimulus animal was a sexually active female but the urge to seek contact was significantly higher when the incentive was a male.
Mating behaviour was possible to activate by TP (0.5 - 1.0 mg/kg s.c.) followed 48 hours later by progesterone (1.0 mg/animal s.c.). Tests were performed 4-6 hours after the progesterone treatment. TP alone was not effective. The optimal time interval between TP and progesterone was 48-72 hours. The possibility that TP is metabolized to oestrogen to excert the effect on the female sexual behaviour is indicated by the fact that the anti-oestrogen ethamoxytriphetol (MER-25,75-150 mg/kg s.c.) inhibited the TP/progesterone activated mating behaviour.

School of Pharmacy, Department of Denmark): THE EFFECT OF ACETALDE- ECTRICAL PROPERTIES OF THE ISOLATED HEART FROM DISULFIRAM-TREATED GUINEA-PIGS IN RELATION TO THE CONTENT OF NORADRENALINE.

Contraction, heart rate and ECG were recorded from guinea-pig hearts perfused with oxygenated Locke sol. in a Langendorff preparation. Continuous addition of acetaldehyde (ACald) to the perfusion fluid increased the heart rate about 2o % and the integrated contraction volume (cardiac output) 5o %. Maximal effect was reached at ACald concentration of 2o μg/ml, further increase to 4o-5o μg/ml caused no further effect. After perfusion with 2o μg/ml the noradrenaline (NA) content increased about 5o % at 2o μg/ml ACald, and decreased towards normal at further increase. Hearts from animals given the dopamin-β-hydroxilase inhibitor disulfiram 25o mg/kg 26 and 2 hours before the experiment showed a decrease of heart NA content from 2.33±o.o5 (n=6) to 1.68±o.o4 (n=6) μg/1oo mg protein. The relative effect of ACald perfusion was the same as in hearts from untreated animals, but an allowed decreased effect was seen with ACald concentration higher than 27 μg/ml. The experiments suggest that ACald has a dual effect: MAO-inhibition and a tyramine like effect. The earl) tachyphylaxis seen in disulfiram treated animals is due to the inhibition of NA-synthesis and a direct depressive effect of disulfiram on the myocardia.

G. Nell*, D. Crüsemann, W. Forth, and W. Rummel (Institute of Pharmacology and Toxicology of the University of the Saarland, 665 Homburg/Saar, W.-Germany): THE NETTRANSFER OF IONS IN THE ISOLATED JEJUNUM UNDER THE INFLUENCE OF DIURETICS.

Diuretics [illegible] absorptive processes in dif[illegible] [illegible]tupp, H.F., Experientia 18, [illegible] [illegible]ics may be used as tools fo[illegible] [illegible]ort processes. According to the method of Fisher and Parsons modified by Rummel and Stupp, isolated segments of the proximal jejunum of the rat were perfused with Tyrode's solution. The nettransfer of ions, water, and glucose under the influence of Mersalyl, Acetazolamid, and Ethacrynic acid, respectively was studied. Mersalyl ($2x10^{-4}$M) causes a decrease of the chloride concentration in the serosal fluid. (Rummel & Stupp). Simultaneously the bicarbonate concentration increases by about 25%. Acetazolamid ($2x10^{-3}$M) increases the chloride concentration in the serosal fluid by 13% and decreases the concentration of bicarbonate by 27%. Ethacrynic acid ($2x10^{-4}$M) diminishes the concentration of bicarbonate in the serosal fluid by 26%. The chloride concentration remains unchanged. In contrast to Acetazolamid Ethacrynic acid inhibits the absorption of fluid by 43% and the transfer of glucose by 52%.

H.-G. Neumann*, H.K. [illegible] [illegible]t für Pharmakologie und Toxiko[illegible] [illegible] Germany): THE ABSORPTION OF [illegible] AND THEIR

Carcinogenic trans-[illegible] [illegible]rans-DAS) and noncarcinogenic cis-4-dimethylaminostilbene (cis-DAS) and 4-dimethylaminobibenzyl (DABB) are readily absorbed after stomach tube feeding in sesame oil. Despite the highly lipophilic properties of these compounds, only 0.35% of administered tritium labeled trans-DAS, 1.3% of cis-DAS and 2.6% of DABB (dose 1 mg) appeared in the thoracic duct lymph within 12 hrs, as shown by radio gas chromatographical analysis. Besides the starting materials, lymph contained several unconjugated and unpolar metabolites. It was not possible to conclude whether these originate primarily from intestinal reabsorption or from peripheral tissues due to the different pattern of these metabolites in bile and blood. To study reabsorption, bile collected from the common bile duct containing a whole mixture of metabolites after application of labeled starting compounds was injected intraduodenally into other animals. The bile of these rats was also collected and a high percentage of radioactivity recovered. Unexpectedly, metabolite analysis showed that the pattern of conjugation and of metabolites in the latter bile was identical to that of the bile administered initially.

The time course of changes in metabolite concentrations in lymph and bile of cannulated animals appears to be considerably different from that in the intact animals.

P.J. Neuvonen*), H.I. Vapaatalo and E. Westermann (Department of Pharmacology, Medical School of Hannover, 3 Hannover - Kleefeld, Roderbruchstr. 101, Germany): SOME METABOLIC EFFECTS OF DOPA AND DOPAMINE IN RATS.

Metabolic effects of L-DOPA and dopamine (DA) were compared with those of noradrenaline (NA) and adrenaline (A) by measuring the changes of plasma free fatty acids (FFA), glycerol, glucose, blood lactate and pyruvate. After i.v. injection of DA lactate and pyruvate were elevated maximally already within 5 min and back to normal levels after 15 min, i.e., at a time when FFA, glycerol and glucose were maximally elevated in plasma. Half-maximal metabolic effects were obtained with 15 µmol/kg of DA i.m., while equimolar doses of L-DOPA proved to be much less active than DA. Similar metabolic effects were obtained with NA although in 20-fold lower doses. Pretreatment with phentolamine (10 or 15 mg/kg) completely prevented the hyperglycaemic response to DA and NA resp., but enhanced the elevation of glycerol without affecting the increase in FFA produced by the amines. Similar effects were observed after pretreatment with dihydroergotamine (10mg/kg). The ß-adrenolytic Kö 592 (50 mg/kg) had no effect on hyperglycaemia but antagonized the increase in FFA and glycerol induced by DA or NA. Pretreatment with pargyline (50 mg/kg), a non-hydrazine inhibitor of MAO, enhanced the elevation of FFA and glycerol induced by DA or NA.

PJN and HIV are fellows of the Alexander von Humboldt Foundation, Germany.

B. Nitz, W. Sturaberg und J. Haan (Pharmakologisches Institut der Philipps-Universität Marburg-Lahnberge, D-355 Marburg/Lahn, Deutschland): DIE WIRKUNG VON BAKTERIELLEM PYROGEN, AMIDOPYRIN UND CHLORPROMAZIN AUF DAS WÄRMEGLEICHGEWICHT DES KANINCHENS (MESSUNGEN MIT DEM BENZINGER-CALORIMETER).

Bei drei verschiedenen Temperaturzuständen, bei 11°, 25°, 29°C wurde die Wirkung von bakteriellem Pyrogen, Amidopyrin und Chlorpromazin sowie deren kombinierte Gabe in einer Calorimeterkammer auf die Rektal-, Ohr-, Hauttemperatur, Atemfrequenz, Herzfrequenz, direkte Wärmeabgabe und CO_2-Produktion beobachtet. Die Calorimeter-kammer wurde in Anlehnung an BENZINGER entwickelt und gestattet auf thermoelektrischem Wege, auch schnelle Änderungen des Wärmebestandes zu messen. In der Kälte verlief die Fieberreaktion schwächer als in der Wärme, hingegen war der temperatursenkende Effekt in kalter Umgebung bei Amidopyrin und Chlorpromazin am stärksten. Die kombinierte Gabe von Pyrogen und Megaphen sowie Pyrogen und Amidopyrin ergab eine stärkere Senkung der Rektaltemperatur als nach Applikation der temperatursenkenden Pharmaka allein In warmer Umgebung, bei 25° und 29°C, war nach Pyrogengabe ein Anstieg der Körpertemperatur zu beobachten, der durch Amidopyrin und Chlorpromazin unterdrückt werden konnte. Die verschiedenen Verlaufsformen der angegebenen Versuche werden durch das differente Verhältnis von Wärmeabgabe und CO_2-Produktion (Wärmebildung) bestimmt.

S. Norn,[x)†] P.A. Shore (Univ.of Texas,Southwestern Med.School,5323 Harry Hines Blvd., Dallas, Texas 75235): BINDING AND LOCATION OF RESERPINE (R).

^{3}H-R,200 μg/kg i.v. and 2 mg/kg i.v.,was injected into rats,and drug levels measured in heart,spleen,adrenal glands,and small intestine. In both cases,all tissues showed a first-order R decline,terminating about 24 to 30 hrs after injection. Following this,a semi-permanent binding persisted for many days. Experiments with high doses of unlabeled R,given 18 or 30 hrs after the labeled drug,reveal that at the 18 hr time,a portion of the drug is reversibly bound,whereas at the 30 hr time,the drug is irreversibly bound. It is suggested that the reversibly bound phase is associated with blockade of the granule amine carrier mechanism,while the irreversible phase is associated with permanent damage to the granular amine storage mechanism. Rats,pretreated with 6-hydroxydopamine (6-HODA) to destruct adrenergic nerve terminals,showed a decrease in noradrenaline content of heart and small intestine by 73 and 67%. Other rats,pretreated with 6-HODA as above,were given ^{3}H-R,200 μg/kg i.v.,and killed 18 hrs later. 6-HODA decreased the ^{3}H-R content in heart and small intestine by 64 and 58%. These results suggest that the majority,if not all,of bound R is localized in adrenergic nerve terminals. In rats,pretreated with ^{3}H-R,200 μg/kg i.v.,stimulation of adrenergic nerve activity by either phenoxybenzamine,insulin or cold stress,and inhibition of the nerve activity by ganglionic blockade (chlorisondamine),didnot,when measured after 24 hrs,affect the R levels in heart,small intestine,spleen and adrenals. Therefore,irreversibly bound R cannot be attached to a granule component releasable by nerve stimulation,but it may be bound to the granule membrane.

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N. Ofori-Nkansah and
stitut der Freien Uni
Germany): SOME METABO
YOSHIDA ASCITES TUMOUR [illegible]

The $^{14}CO_2$-production from 1-^{14}C-glucose in Yoshida ascites-tumour cells is dose dependently inhibited, if incubated in vitro in the presence of 6-aminonicotinamide or after 10, 25, or 50 mg/kg body weight administered intraperitoneally into tumour bearing mice 9 hours before. In homogenates of ascites tumour cells pretreated with the drug (50 mg/kg body weight) about a 70-fold increase in the content of 6-phosphogluconate parallelled by a 3-4-fold increase in the content of glucose 6-phosphate is observed. The content of glucose 1-phosphate and fructose 6-phosphate increase concomitantly. Glyceraldehyde 3-phosphate and dihydroxyacetone phosphate increased 6-7-fold with a concomitant 5-6-fold increase of fructose 1,6-diphosphate. The contents of glycerine 3-phosphate, pyruvate, lactate, malate, and ATP decreased; that of phosphoenolpyruvate is slightly raised. The accumulation of the metabolites is dose and time dependent and is totally or partly inhibited by nicotinamide (50 mg/kg body weight). The cells show ballooning of the rough endoplasmic reticulum and of the perinuclear cistern. The data presented suggest inhibition of the glycolysis and of the Pentose Phosphate Pathway in ascites tumour cells by in vivo synthesized NAD(P)-analogues of 6-aminonicotinamide as seen in the brain of rats pretreated with the drug (Herken et al. 1969a,b; Herken 1970; Lange et al. 1970).

K. Breiter and F.K. Ohnesorge[+] (Department of Pharmacology, Hospitalstr. 4-6, 23 Kiel, W.Germany): ON THE ENTERAL ABSORPTION OF TERTIARY AND QUATERNARY AMINES FROM THE ISOLATED GUINEA PIG JEJUNUM.

The lumen of isolated sections of gut, taken from the upper part of the jejunum, was perfused with oxygenated Krebs-Henseleit-solution at different pH-values. The absorption of ^{14}C- and ^{3}H-labelled amines (Arecaidinisopropylester, Dihydroarecaidinisopropylester-iodomethylate, atropinesulphate, N-Methyl-atropine-iodide, Bisacodyl, N-Methylbisacodylmethylsulphate and Hexamethonium) from the inner and outer surface of the gut was determined. The rate of absorption was expressed as percentage of the concentration of the perfusate/ h x 10 mg dry weight of the perfused section.

The rate of absorption of the tertiary compounds ranges from 8-10 %/h x 10 mg of the nonionized fraction of the drugs with exception of Bisacodyl. The quaternary compounds were only absorbed between 0.5-1%/h x 10 mg independent on changes of the pH of the perfusate. - Comparing the absorption rates of the inner to the outer surface and vice versa, the rates were found to be identical. There is no evidence for an active transport process. It is concluded, that the absorption is mediated by passive diffusion, with different pathways for the ionized and nonionized fractions of the drugs investigated.

H. Osswald,[x)] and G. Fülgraff, (Abt. Pharmakologie der Med. Fakultät, TH Aachen, Medizinisch-Theoretische Institute, 51 Aachen, Germany) DETERMINANTS OF RENAL ETHANOL EXCRETION.

The renal excretion of ethanol has been studied in dogs employing the technique of a single rapid injection of ^{14}C-ethanol together with a glomerular marker (Creatinine) into one renal artery and collection of urine samples at 10-20 sec intervals separately from both ureters. ^{14}C appeared in the urine within 10 sec or less after the injection, whereas creatinine appeared after a delay of 30-90 sec. This suggests that ethanol may enter the urine in the most distal parts of the nephron. The percentage of ^{14}C-ethanol of the injected amount excreted by the kidney is extremely low compared with creatinine. Experiments in different states of diuresis, however, indicated that ethanol excretion depends quantitatively on urinary volume and osmolality It is assumed that ethanol can diffuse readily from tubular cells into intratubular fluid or blood and vice versa following its concentration gradients and that ceteris paribus flow velocity of blood and tubular fluid determines quantitatively ethanol excretion.

S. Guimarães and W. Osswald[x] (Laboratório de Farmacologia, Faculdade de Medicina, Porto, Portugal). THE EFFECTS OF COCAINE AND DENERVATION ON THE RESPONSES OF ISOLATED VEIN STRIPS TO NORADRENALINE.

The sensitivity to noradrenaline and its termination of action in isolated strips obtained from the lateral saphenous vein of the dog, as well as the capacity of the strips to remove noradrenaline from the incubation medium was studied in normal, surgically denervated and cocaine pretreated strips. Cocaine (2 ug/ml) increased noradrenaline sensitivity 7.5-fold, augmented the time needed for half-relaxation after oil-immersion to 2.37 times that of controls and reduced noradrenaline removal (0.86 ± 0.15 ug/g/hr after cocaine, controls 1.99 ± 0.13 ug/g/hr). Denervated strips showed a 5-fold increase in sensitivity to noradrenaline, had a half-relaxation time of 2.24 times that of controls and removed 0.55 ± 0.12 ug/g/hr. In no instance did cocaine differ significantly from denervation. Excellent correlations between noradrenaline content and removal capacity, sensitivity to noradrenaline, inactivation capacity and potentiation by cocaine were found. On the whole, the results give evidence for a prejunctional site of action of cocaine.

K. Fredricson Overø (Research Laboratories, H. Lundbeck & Co. A/S, Ottiliavej 7-9, 2500 Copenhagen) A MODIFICATION OF THE ^{3}H-ACETIC ANHYDRIDE COUPLING METHOD WITH INCREASED SPECIFICITY FOR SECONDARY AMINES.

The ^{3}H-acetic anhydride method of Hammer and Brodie (J. Pharmac exp Ther. 1967, 157, 503-508) has been widely used during recent years for assay of primary and secondary amines. Especially for pharmacokinetic studies of a secondary amine the lack of specificity may be a drawback, since the corresponding primary amine might be present as a metabolite

For this reason the possibility of masking primary amines by coupling with salicylic aldehyde as presented by Nyberg (J. Pharm. Pharmac. 1970, 22, 500-506) has been investigated. The presence of salicylic aldehyde (0.1 M) during the first extraction step of the method was found to reduce considerably the amount of primary amine measured as ^{3}H-amide. Secondary amines were only slightly influenced. Thus increased specificity for secondary amines was achieved by this minor change in the method.

The practical importance of the modification was illustrated by experiments on plasma from rats given nortriptyline and humans given Lu 5-003, a bicyclic thymoleptic. In the former case the modified method is of less importance - in the latter case, however, it would be advantageous.

V. Pedersen[x] and A.V. Christensen (Department of Pharmacology, H. Lundbeck & Co. A/S, Copenhagen, Denmark): METHYLPHENIDATE ANTAGONISM IN MICE AS A RAPID SCREENING TEST FOR *NEUROLEPTIC DRUGS.*

By chance it was observed that methylphenidate in mice induces an intense gnaw-compulsion syndrome. Mice were given methylphenidate (60 mg/kg s.c.) and were placed in special *cages*, *two mice in each cage, for one hour. The cages were* placed on corrugated paper and within 10-15 min. the mice began to bite the paper. The gnaw-compulsion syndrome was studied in mice pretreated with H 44/68 (d,l-α-methyltyrosine-methylester hydrochloride, 200 mg/kg i.p. 4 h before test). H 44/68 completely abolished the compulsive gnawing. *L-DOPA* (200 mg/kg i.p. 1 h before testing) completely reactivated the mice pretreated with H 44/68. Furthermore it was shown that pretreatment with sodium diethyldithiocarbamate (three doses of 500 mg/kg i.p., 6, 4 and 1 h *before testing) hardly affected the compulsive gnawing.* The results indicate that the effect of methylphenidate is dependent on the presence of dopamine. A number of neuroleptics was tested for antagonistic effect and the following order of potency was obtained: fluphenazine > haloperidol > flupenthixol > clopenthixol > chlorprothixene > chlorpromazine. The methylphenidate antagonism test seems to be a rapid and reliable method for classification of neuroleptic compounds.

O.Pelkonen[x], P.Jouppila, M.Vorne and N.T.Kärki (Department of Pharmacology, University of Oulu, Finland): EFFECTS OF MATERNAL INTAKE OF PHENOBARBITAL AND CIGARETTE SMOKING ON DRUG METABOLISM IN HUMAN FOETAL LIVER AND PLACENTA.

Human foetal liver (Pelkonen et al.: Acta Phys.Scand. 1969, 69, suppl. 330 and Acta Pharmacol.Toxicol. 1971, 29, 284) and human term placenta (Welch et al.: Clin.Pharm.Ther. 1969, 10, 100) are able to metabolize foreign compounds.
In this work we studied the effects of phenobarbital (Phe) intake and cigarette smoking (CS) on the capability of human foetal liver and placenta homogenates to metabolize 3,4-benzpyrene (BP) and N-methylaniline (MA).
Phenobarbital intake (100 mg/day for 7-25 days)

Foetal liver		MA (umol/g/h)	BP (nmol/g/h)
CONTROL	n=10	0.65±0.18 (S.D.)	0.192±0.051
PHE	n=10	0.81±0.16	0.229±0.040
significance		p<0.05	0.10>p>0.05

Phe had no effect on MA or BP metabolism in immature or term placental homogenates.
Cigarette smoking (average 9.5 cigarettes/day)

Placenta			MA (umol/g/h)	BP (nmol/g/h)
IMMATURE	CONTROL	n=19	0.07±0.11	not detected
	CS	n=11	0.10±0.11	0.076±0.086
TERM	CONTROL	n=11	0.13±0.09	not detected
	CS	n=11	0.12±0.15	0.254±0.281

CS had no effect on BP or MA metabolism in foetal liver.

H Aulepp H D Peters[x], K Karzel, F !
sches Institut der Universität Bonn Re

ON THE INFLUENCE OF ANTIPHLOGI
DERIVATIVES ON SOME METABOLIC ...
IN VITRO

In previous investigations a series of antiphlogistic drugs was found to depress the multiplication of various types of cells cultured in vitro The aim of the present experiments was to see wether the phenylalkylacid-derivatives ibuprofen (1) bufexamac (2) and alclofenac (3) possess similar properties and if so wether they influence metabolic parameters of fast growing cells The experiments were performed on suspension cultures of an established strain of Ehrlich ascites tumor cells Under standardized conditions the cells were incubated for 24 hrs with different concentrations of the test-substances so that the cell multiplication was inhibited by 15 50 and 85 % Before and after incubation the following parameters were determined (a) cell number (b) mean cell volume (c) protein DNA- and RNA content of 10^5 cells The data were evaluated statistically by means of the regression and correlation analysis Until now the following results have been obtained cytostatic activities were observed in the following concentrations (1) 2 0 - 0 1 (2) 0 2 - 0 02 (3) 2 0 - 0 1 mM The RNA content of the cells was statistically significant and dose dependent influenced by (1) and (2) The protein content of the cells showed similar changes but the correlation was not as sharp as in the case of RNA while the DNA content was not obviously changed

C Peterson* and B Diamant (Dept. of Pharmacology, Karolinska Institutet, Stockholm, Sweden): OBSERVATIONS ON ENERGY METABOLISM OF ISOLATED RAT MAST CELLS AND HISTAMINE RELEASE INDUCED BY ATP AND 48/80.

Histamine release from isolated rat mast cells ($2\text{-}3\times10^3$ cells/ml) induced by exogenous ATP ($3\text{-}5\times10^{-5}$M) in the presence of Ca^{++} was enhanced by glucose and mannose but not by fructose or galactose (Peterson C and Diamant B Acta physiol. scand. 1969 suppl. 330 p. 61) Pyruvate and lactate also enhanced the histamine release. Iodoacetate (10^{-4}M) in the absence of added metabolic substrates partially inhibited the histamine release. The inhibition was counteracted by pyruvate and lactate but not by glucose or mannose. Antimycin A (10^{-7}M) completely blocked histamine release irrespectively of the addition of substrates As judged from the production of lactate in concentrated samples of mast cells ($3\text{-}4\times10^6$ cells/ml) only glucose and mannose were metabolized through the Embden-Meyerhof pathway. Lactate accumulation in the presence of glucose and mannose was abolished by iodoacetate whereas it was enhanced by antimycin A. In the absence of added hexoses a slight accumulation of lactate could be registered in the presence of antimycin A The addition of increasing concentrations of ATP ($>2\times10^{-5}$M) to samples of concentrated mast cells gradually decreased the lactate accumulation induced by glucose. Antimycin A did not influence this decrease although it markedly inhibited the histamine release induced by ATP In contrast, in the presence of glucose, antimycin A did not inhibit 48/80-induced histamine release. 48/80 did not affect the accumulation of lactate caused by glucose metabolism in rat mast cells. The results suggest that histamine release induced by ATP depends on an intact oxidative phosphorylation and that ATP in contrast to 48/80 decreases glycolytic lactate formation in the cells.

A. Philippu[+)] and H. Przuntek (Department of Pharmacology, University of Würzburg, Germany): EFFECT OF DRUGS ON THE RISE OF BLOOD PRESSURE DUE TO STIMULATION OF THE HYPOTHALAMUS.

The third ventricle and the aqueduct of the anaesthetized cat were cannulated and the hypothalamus was superfused with artificial cerebrospinal fluid. Electrical stimulation of the n. posterior of the hypothalamus for a period of 30 sec (10 V, 40 HZ, 6 msec) elicited a rise of the blood pressure of 58±3 mm Hg. The electrical stimulation was repeated every 10 min. Superfusion of the hypothalamus with bretylium tosylate ($5x10^{-3}$M) caused a gradual and irreversible impairment of the rise of the blood pressure during stimulation of the n. posterior. The inhibition achieved its highest rate (47% of the control values) 120 min after beginning the superfusion with bretylium. Tetracaine ($5x10^{-3}$M) also diminished the rise of the blood pressure (20% of the control values after 70 min). Pretreatment of cats with 6-hydroxydopamine (3x1 mg/kg were applicated by means of a cannula implanted into the lateral ventricle) provoked a decrease of the noradrenaline content of the hypothalamus and of the rest-brain (30% and 47% of the control values respectively) and furthermore an impairment of the rise of the blood pressure during stimulation of the n. posterior (33±3 mm Hg. $p < 0.001$). The blood pressure of the anaesthetized cats was not affected by 6-hydroxydopamine. The results favour the assumption that the rise of the blood pressure during electrical stimulation of the n. posterior of the hypothalamus is mediated by noradrenergic neurones of the brain.

K.H.Rahn[x)] and W.Rupp (II.Medizinische Klinik, Universität Mainz and Medizinisch-Wissenschaftliche Abteilung, Farbwerke Hoechst AG, Frankfurt/Main, Germany): PLASMA LEVELS AND URINARY EXCRETION OF GUANETHIDINE IN HYPERTENSIVE PATIENTS.
In 5 patients with essential hypertension and serum creatinine levels below 1.5 mg%, the plasma levels of guanethidine (ismelin[R]) as well as the urinary excretion of the drug were studied after i.v. injection of 12.1 mg ^{3}H-guanethidine (o.1 mc). Experimental data on guanethidine plasma levels as analyzed by means of a computer program did fit best with a 3 compartment model. Within 1o days, 89 ± 3.3% of the radioactivity given to the patients was excreted in urine, 61 ± 6.5% of it in form of the parent drug. Four other hypertensives with chronic renal failure (serum creatinine 4-6 mg%) excreted 61 ± 4.8% of the radioactivity within 1o days after i.v. injection of ^{3}H-guanethidine. In these patients, 33 ± 2.8% of the radioactivity found in urine was excreted as unchanged drug. Thus, guanethidine is excreted more slowly and metabolized to a greater degree by patients with renal failure.

O.Rohte (AB Leo, Helsingborg, Schweden): TIERPHARMAKOLOGISCHE VERSUCHE MIT LEO 1028, EINER *NEUEN SUBSTANZ MIT ANTIPHLOGISTISCHEN*, ANALGETISCHEN UND ANTIPYRETISCHEN EIGENSCHAFTEN.

Leo 1028 = 2-ethyl-2-methyl-6-chlor-1,3-benzdioxol-5-yl)-azetyl-saure. Wenn nicht anders angegeben, alle Werte = mg/kg p.o. Abkürzungen: M = Maus, R = Ratte. *LD50, 48 Std.*, M

[illegible] i.v. Grenzwert Pfoten- [illegible] rrageenan 13; *Granuloma* [illegible] u. Sekretion durch 7x80. [illegible] d 15x80 signifikante Ver- [illegible] Reduktion der Schmerzreak- [illegible] nzgeschwüre, Verbesserung [illegible] olinödem bei adrenal. R: [illegible] zwert Randall-Selitto R: [illegible] Dosis: 32. *ED50 f.* Auf- [illegible] h Bradykinin, Konzett-Rössler, Meerschw.: 0.57 i.v. Verlängerung der Hexobarbital-M u. Alkoholschlafzeit M durch 32 bzw. 80. Dämpfung der Putzinstinkte M: 80. Hemmung der Kampflust M: 200. Magenblutungen R erst durch 200. Durch 80 intraduodenal bei Katzen keine Veränderungen des Blutdruckes, der NA- u. Tyraminreaktionen. *Carotiss. Reflexe gedämpft. El-Schock-* u. Strychninkrampf M: 200 ohne Wirkung. 200 reduzieren Letalität *im Pentetrazoltest* M. *Bedingte Refl.* R und *Reser-*pinhypothermie M durch 200 nicht beeinflusst. EK50 für Entkopplung oxydat. Phosphor.: 0.36 mM.
In den Versuchen wurde Leo 1028 mit Phenylbutazon, Indomethacin, Aminopyrin und Acetylsalicylsäure verglichen.

H.ROOS and K.PFLEGER (Inst.f.Pharmakol.& Toxikol.Homburg/Saar, W.-Germany) KINETICS OF ADENOSINE UPTAKE AND THE INFLUENCE OF DIPYRIDAMOLE.

Adenosine is taken up very fast by heart muscle cells, erythrocytes, lungs and other organs (JACOB,M.I. and BERNE,R. Am.J.Physiol. 198, 322, 1960; KOSS et al., Arzneim.Forsch. 12, 1130, 1962; PFLEGER et al., Biochem. Pharmacol. 18, 43, 1968). This uptake can be inhibited by the coronary dilatators dipyridamole, hexobendine and lidoflazine (PFLEGER et al., Arzneim. Forsch. 19, 1972, 1969). The uptake in red cells can be described by the following equation:

$$Y = \frac{V}{1 + \frac{K_m}{S}} + P \cdot S \; *$$

* Y=uptake; V and K_m are the usual terms of the Michaelis-Menten equation; P is a constant and S is the concentration of the subtrate.

The validity of the equation can also be shown for inosine, methyladenosine, and dimethyladenosine. This is a further proof for the existence of two ways of uptake. The results will be discussed in connection with the mode of action of dipyridamole in coronary dilatation.

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METABOLIC FATE OF CANNABINOIDS IN RABBIT AND RAT:

Δ1-tetrahydrocannabinol-C^{14} (Δ1-THC-C^{14}), synthetically prepared, was shown to be rapidly distributed in the body after i.v. injection in the rabbit, the half-life of radioactivity in blood being about 15 min. The distribution of radioactivity in tissues reflected the elimination of Δ1-THC through liver and kidneys. Brain and spinal cord showed the lowest activity levels of all the investigated tissues. Three days after administration high levels of radioactivity still persist in body fat and spleen. After i.v. injection in the rabbit, Δ1-THC was rapidly converted to more polar metabolites, at least two of them appearing in the urine. Δ1-THC was also metabolized in vitro with 10.000 g supernatant from rabbit liver. By MS and NMR spectrometry the major metabolite was identified as 7-hydroxi-Δ1-THC. Experiment in mice showed that this compound is an active metabolite, the same as an ethersoluble metabolite found in liver half an hour after i.v. injection of Δ1-THC in the rabbit. 7-hydroxi-Δ1-THC is not excreted as such in the urine of the rabbit. It is conjugated and also probably further oxidized to the corresponding acid before excretion. The binding of Δ1-THC and 7-hydroxi-Δ6-THC to human plasma proteins has been investigated in vitro by different electrophoretic techniques.

B. Schoene,* R. A. Fleischmann, H. Remmer and H.-F. v. Oldershausen
(Institut für Toxikologie, Medizinische Klinik, Tübingen, Germany):
THE CYTOCHROME P-450 CONTENT AND THE ACTIVITIES OF VARIOUS MICROSOMAL ENZYMES IN NEEDLE BIOPSY MATERIAL FROM HUMAN LIVER.

Micromethods have been developed for determining microsomal, particularly drug-metabolizing, enzymes in about of 20 mg of needle biopsy material from human liver. Since investigations in homogenates and microsomes of rat liver showed comparable results and because of the small amount of tissue available, we did not isolate microsomes and used liver homogenate as enzyme scource for measuring the amount of cytochrome P-450 and the activity of the following enzymes: NADPH-dependent cyt.-c-reductase, glucose-6-phosphatase, pseudocholinesterase and the o-demethylase with p-nitroanisol as substrate. The normal human liver and of patients with mild types of hepatitis contains 11 ± 3 nmoles P-450 per 1 g liver wet weight, about one third of the rat liver's content. This is one of the reasons for the slower speed of drug-oxidation in man. Out of 50 patients studied, the liver of 6 patients with severe hepatitis or cirrhosis showed a 50 % lower P-450 content and a slow oxidation rate. About 50 % more P-450 has been found in the liver of 4 patients receiving various drugs which are probably enzyme inducers. Drugs well known for their capacity to induce enzymes, produced a threefold increase of P-450 in 3 patients. In post mortem liver the P-450 content was more than 50 % reduced compared with the normal values. The variation of the other enzymes investigated is much smaller and indicates that liver injury and enzyme induction affects cyt. P-450 much more than the other microsomal enzymes studied.

J Scholtholt[+]
la Farbwerke
NISMUS ZWI
POTENZIERENDEN KORONAR...

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dig erhalten

H. Scholz (Pharmakologisches Institut der Universität, Obere Zahlbacher Str. 67, 65 Mainz, Germany) THE POSITIVE INOTROPIC ACTION OF DIBUTYRYL CYCLIC AMP AT DIFFERENT EXTRACELLULAR CA CONCENTRATIONS.

In isolated electrically driven (2 Hz) rat left atria the monosodium salt of the N^6-2'-O-dibutyryl derivative of cyclic adenosine-3',5'-monophosphate (DB-AMP) produced a concentration-dependent positive inotropic effect. At 1 8 mM Ca, the threshold concentration was $5 \cdot 1o^{-5}$ M, the peak concentration ($1o^{-3}$ M) augmented contractile force to 2o3 % of control values. The effect of the drug was due to an increase in the rate of tension development, the time to peak tension and the duration of contraction remained unchanged The time course and the magnitude of the inotropic effects of DB-AMP were dependent on the $[Ca]_e$ The increment in myocardial contractility caused by $1o^{-3}$ M DB-AMP developed faster at high than at low $[Ca]_e$. On the other hand, the positive inotropic effect of $1o^{-3}$ M DB-AMP was most evident at normal (1 8 mM) and reduced (o 45, o,9 mM) $[Ca]_e$, less pronounced at 3 6 mM Ca and was insignificant at 7 2 mM Ca In this respect, the action of DB-AMP resembles that of adrenaline and theophylline, the positive inotropic effects of which have been thought to be mediated by cyclic AMP (see Kukovetz & Pöch Arch Pharmak. 197o, 266, 236, Skelton et al. Circulation Res 197o, 26, 35)

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B Schoene,† R A Fleischmann, H Remmer and H F v Oldershausen (Institut für Toxikologie, Medizinische Klinik, Tubingen, Germany):
THE CYTOCHROME P 450 CONTENT AND THE ACTIVITIES OF VARIOUS MICROSOMAL ENZYMES IN NEEDLE BIOPSY MATERIAL FROM HUMAN LIVER

Micromethods have been developed for determining microsomal, particularly drug metabolizing, enzymes in about of 20 mg of needle biopsy material from human liver Since investigations in homogenates and microsomes of rat liver showed comparable results and because of the small amount of tissue available, we did not isolate microsomes and used liver homo genate as enzyme source for measuring the amount of cytochrome P 450 and the activity of the following enzymes NADPH-dependent cyt -c-reductase, glucose 6 phosphatase, pseudocholinesterase and the o-demethyl ase with p nitroanisol as substrate The normal human liver and of patients with mild types of hepatitis contains 11 ± 3 nmoles P 450 per 1 g liver wet weight, about one third of the rat liver's content This is one of the reasons for the slower speed of drug oxidation in man Out of 50 patients studied, the liver of 6 patients with severe hepatitis or cirrhosis showed a 50 % lower P 450 content and a slow oxidation rate About 50 % more P-450 has been found in the liver of 4 patients receiving various drugs which are probably enzyme inducers Drugs well known for their capacity to induce enzymes, produced a threefold increase of P 450 in 3 patients In post mortem liver the P 450 content was more than 50 % reduced compared with the normal values The variation of the other enzymes investigated is much smaller and indicates that liver injury and enzyme induction affects cyt P-450 much more than the other microsomal enzymes studied

S.O. Olsson and J. Schrold[x] (Research Laboratories Ferrosan Malmo and Copenhagen): A COMPARISON OF FG 5310 A NEW SELECTIVE MONOAMINE OXIDASE INHIBITOR AND OTHER MAO INHIBITORS ON THE BLOOD PRESSURE RESPONSE TO TYRAMINE

It has been shown that FG 5310 γ morpholino p aminobutyrophenone is a strong reserpine antagonist and a selective MAO inhibitor in vivo Therefore the tyramine potentiating effect of FG 5310 was compared to some non selective MAO inhibitors Rats were treated one or several times orally with the MAO inhibitors in doses which protect against reserpine Tyramine was administered intraduodenally (i d) to pithed or anaesthetized rats or orally to unanaesthetized rats The blood pressure response was measured intra-arterially and compared with control rats tested in the same manner as the treated rats Similar experiments were done using anaesthetized cats

The pressor effect of 5 mg/kg tyramine i.d. to rats

Control rats	FG 5310 (2-50 mg/kg)	Tranylcypromine (2 mg/kg) or Isocarboxazid (5 mg/kg)
0 8 mmHg	5 20 mmHg short dur	15-75 mmHg long duration

The maximal mean arterial pressure (1) before and after 1 mg/kg tyramine i.d to cats

N	Control	N	FG 5310 (50 mg/kg)	N	Tranylcypromine (10 mg/kg)	
8	115±22 mmHg	7	130±22 mmHg	7	122±27 mmHg	Before
8	119±19 mmHg	7	137±22 mmHg	7	187±22 mmHg	After
			P>0 05(2)		P<0 005(2)	

It is concluded that FG 5310 has a much weaker tyramine potentiating effect than several non selective MAO inhibitors

(1) ½ x (Systolic + Diastolic Pressure) (2) Student s t test

J. Schuberth and A Sundwall (Psychiatric Research Center, Ulleråker Hospital, Uppsala and Department of Pharmacology, Umeå, Sweden): A METHOD FOR THE DETERMINATION OF CHOLINE IN BIOLOGICAL MATERIALS

In the study of acetylcholine (ACh) metabolism choline (Ch) is of considerable interest since this compound is the precursor as well as the metabolic end-product of ACh. The principle of the Ch assay procedure, presented in this communication, is based on the acetylation of Ch to ACh with ^{3}H-acetyl-CoA in the presence of choline acetyltransferase prepared from human placenta (Schuberth, J Biochem Biophys. Acta, 1966, 122, 470). The ^{3}H-ACh formed after incubation is isolated by high voltage paper electrophoresis and the amount of Ch originally present in the sample can be measured as radioactivity By this procedure 20 µl samples containing 0 5 nmoles/ml of Ch can be assayed. It was controlled that Ch-containing compounds (lecithin, phosphoryl Ch, cytidyldiphosphoryl Ch and glycerophosphoryl Ch) did not interfere with the assay to any appreciable extent. The specificity of the method was also demonstrated by the complete hydrolysis of the ^{3}H-ACh with acetylcholinesterase prior to paper electrophoresis The following normal values were found (nmoles/ml± s): human plasma 10.9±0 43 (10), dog plasma 10.5±0 55 (23), mouse plasma 15.0±0.87 (17), human lumbar spinal fluid 2 2±0.10 (10), dog ventricular CSF 4.6±0 25 (10) dog cisternal CSF, 1.9±0 26 (7) and whole mouse brain 68.1±6 11 (10) nmoles/g.

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ANALYSIS OF BIOLOGICAL FLUIDS

Recently it was demonstrated that intake of cannabis resin could be detected by thin-layer chromatography of urine after the conversion of one or more metabolites to cannabinole (CBN) and tetrahydrocannabinole (THC) by n-toluenesulfonic acid treatment (same authors Acta pharmacol et toxicol 1971, 29 111-112) By chloroacetylation the CBN and the THC can be transformed into compounds which can be measured with high sensitivity by gas chromatography using an electron capture detector The sensitivity of the gas chromatographic procedure allows 0 04 ng of delta-9-THC to be measured Based upon this method, experiments on the biological disposal and metabolism of intravenously administered delta-9-THC are in progress

E Schraven[+] and R E Nitz (Medizinische Forschung Cassella Farbwerke Mainkur AG , Frankfurt/Main, BRD) STUDIES OF THE TRANSPORT OF GLUCOSE INTO THE BRAIN

Glucose is nearly the only energy-support of the brain. It is almost completely transported by an active machanism across the blood-brain barrier. Quadbeck demonstrated, that the uptake of glucose is influenced by several substances. We studied in a similar model with mice the influence of blood glucose level upon the uptake of radioactivity into the brain after i v -injection of U-C^{14}-glucose We can show, that there is a highly significant correlation between blood level of glucose and uptake of radioactivity into the brain Lowering of blood glucose, for example with insulin, resulted in an elevation of the uptake of radioactivity and vice versa an increase of the glucose level, for example by glucagon, led to a diminished rate of uptake Supposing that independent from the blood glucose level the quantity of glucose transported across the blood-brain-barrier (active transport) is always the same, we calculated the uptake of radioactivity in relation to the blood glucose concentration We can demonstrate, that the calculated and the experimentally obtained data are exactly corresponding over a wide range

QUADBECK,G Munch Med Wschr 104, 24 (1962)

U.Seib[x], H.H.Wellhöner, and B.Hensel (Pharmakologisches Institut, Rudolf Buchheim-Str. 4, Giessen, Germany):
THE INFLUENCE OF REGIONAL MUSCULAR ACTIVITY ON THE ASCENSION OF TETANUS TOXIN IN THE SCIATIC NERVE.

Tetanus toxin labelled with J^{125} (MLD/kg mouse: 7.5ng; γ radioactivity (γA): 85×10^{10} cpm/g toxin, immune reactivity 85%) was injected into the gastrocnemius muscles (GS) of cats (1 or 2×10^{6} cpm/kg cat, equivalent to 1.2 or 2.4 μg toxin/kg cat; calculated MLD/Kg cat: 4.5 μg toxin). The cats were killed 24 h later, tissue probes were taken and their γA was measured. Main results. 1. Unanesthetized cats injected into the right GS only: The γA is elevated in the right L7 and/or S1 spinal cord segment and is there 9.9 ± 1.3 times higher than on the left side. This quotient right/left (Qr/l) is reduced to 3.3 ± 0.8, if the cat is maintained in general anesthesia throughout the experiment. 2. No regional elevation of γA is observed after injection of NaJ^{125} into the GS. 3. Anesthetized cats, both GS injected, both hamstring nerves cut between ligatures, right GS directly stimulated with trains of square wave impulses. The Qr/l is 3.7 ± 0.4. 4. Cats as in 3., but peripheral stump of right hamstring nerve stimulated instead of GS. The Qr/l is 0.98 ± 0.03. 5. Cats as in 3., but right hamstring nerve intact and right sciatic nerve (including hamstring part) stimulated: The Qr/l is 1.50 ± 0.06. 6. Unanesthetized cats, both GS injected, left hamstring, obturator and femoral nerve ligated (ether anesthesia) The Qr/l is 1.28 ± 0.03.

P. Sen[x], A Goth and N Chakravarty (Dept. of Pharmacology, University of Odense, Denmark and Dept. of Pharmacology, Southwestern Medical School, University of Texas, Dallas, Texas, U.S.A.) POTENTIATION OF DEXTRAN-INDUCED HISTAMINE RELEASE FROM RAT MAST CELLS BY PHOSPHATIDYL SERINE.

Histamine is readily released by dextran in vitro from mixed peritoneal cells of Wistar rats, but little or no release occurs from isolated pure populations of mast cells. The response of the isolated mast cells can, however, be restored by adding phosphatidyl serine to the medium prior to exposure to dextran. The dextran-induced histamine release from mixed peritoneal cells is also potentiated by phosphatidyl serine but not by phosphatidic acid or l-serine. Serum albumin and ficoll, generally used for the isolation of mast cells, have an inhibitory effect on histamine release induced by dextran. Conceivably, phospholipids in the mast cell membrane are involved in the histamine release process. The methods employed for the preparation and isolation of the cells may cause changes in the mast cell membrane - specially in membrane phospholipids, leading to a diminished response or lack of response of the mast cells to dextran. Phosphatidyl serine appears to counteract this effect and thus restore the responsiveness of the cells to dextran-induced histamine release.

B. Schölkens (Abteilung für Pharmakologie, Farbwerke Hoechst A.G. 6230 Frankfurt (M) 80 Germany): ÜBER DIE KARDIOSTIMULATORISCHE UND ANTIARRHYTHMISCHE WIRKUNG VON SEKRETIN UND GLUKAGON!

Wegen der grossen Strukturähnlichkeit der beiden Polypeptide Sekretin, von den Zellen der Duodenalmucosa gebildet, und Glukagon, von den α-Zellen des Pankreas stammend, wurden vergleichende Untersuchungen an Hunden, Katzen, Rhesusaffen, Schweinen, Schafen und Meerschweinchen durchgeführt. Nach i.v.-Injektion von 1-10 E/kg Sekretin respektive 1-50γ/kg Glukagon fanden sich beim Hund: Steigerung der Herzfrequenz; Zunahme des Durchflusses in Aorta, linker Koronararterie und Arteria femoralis. Abfall des peripheren Widerstandes. Steigerung des Herzminutenvolumens, des Schlagvolumens und der Herzleistung. Zunahme der Kontraktilität. Durch Herzglykosidintoxikation erzeugte ventrikuläre Rhythmusstörungen wurden durch 10-50 E Sekretin i.v. bei Katzen und Rhesusaffen oder durch 50γ/kg Glukagon i.v. bei Hunden und Rhesusaffen behoben. Im Gegensatz zu Sekretin war durch Glukagon eine ausgeprägte Toxizitätsminderung von K-Strophanthin bei der Katze zu erzielen. Bei Schafen liess sich weder ein positiv inotroper noch ein positiv chronotroper Effekt nach beiden Substanzen beobachten. Beta-Rezeptorenblockade führte zu keiner Hemmung der Sekretin- und Glukagonwirkung. Neben der positiven Chronotropie und der positiven Inotropie haben Sekretin und Glukagon auch eine antiarrhythmische Wirkungskomponente gemeinsam. Zwischen der Herz-Kreislauf-Wirkung von Sekretin und Glukagon findet sich eine weitgehende qualitative Parallelität.

[illegible] (Institute of Medical [illegible] Denmark): THE EFFECT [illegible] (CENTYL®) ON CALCIUM METABOLISM IN RATS.

A reduction of renal calcium excretion during thiazide administration has been observed in patients and normal persons. The mechanism responsible for this effect is unknown. Bendroflumethiazide (Centyl®) was administered to intact, parathyroidectomized (PX), thyro-parathyroidectomized (TPX) and thyroidectomized (TX) rats in order to investigate the role of parathyroid hormone and calcitonin in the reduction of urinary calcium excretion seen during thiazide administration. A significant reduction in renal calcium excretion was observed in all groups suggesting that the effect of thiazides on renal calcium excretion is independent of the two most important calcium regulating hormones. In one aspect the intact rats differed from the PX-rats. Thus renal calcium excretion increased after cessation of the thiazide administration whereas renal calcium excretion in PX-rats after a few days attained the basal level, which was never exceeded. This suggest a compensatory hypersecretion of parathyroid hormone in intact rats. In order to elucidate the effect of bendroflumethiazide administration on calcium metabolism the effect on bone metabolism was investigated in rats pre-labelled with ^{45}Ca. An increased bone concentration of ^{45}Ca (and an increased specific activity) was found in the thiazide group suggesting that bone resorption was reduced during bendroflumethiazide administration.

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B. Sjöqvist, J.W. Dailey and E. Anggård (Department of Pharmacology, Karolinska Institutet, S-104 01 Stockholm 60, Sweden). NEW GAS CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF HOMOVANILLIC ACID IN URINE AND CEREBROSPINAL FLUID.

Homovanillic acid (HVA) is the main metabolite of dopamine. The analysis of this compound in biological fluids can be accomplished by new gas phase analytical methods. HVA in urine was analyzed by extraction into ether, silicic acid chromatography, conversion to the methyl ester-methyl ether derivative and GLC analysis on an 1 % XE-60 column using 4-hydroxy-3-methoxyphenylpropionic acid ($HVA-CH_2$) as an internal standard. The method permits accurate (±3.7 %) determination of levels of HVA in normal human urine.

From cerebrospinal fluid (CSF) HVA was extracted into ethylacetate at an acidic pH, followed by preparation of the methyl ester-heptafluorobutyric derivative. After a final purification on lipophilic Sephadex (Reversex) HVA was measured by GLC with an electron capture detector on a 3 % QV-1 column. Quantitation was achieved by internal standardization with $HVA-CH_2$. The analytical precision was ±5.5 % using 2 ml of normal lumbar CSF. An alternative procedure with higher sensitivity entailed the use of deuterium labelled HVA-Me as an internal standard followed by analysis with GLC-mass spectrometry.

L. Albanus, M. Jönsson, B. Sparf* and J. Vessman (Research Department of the KABI Group, Stockholm, Sweden). A STUDY OF THE INDUCTION EFFECT OF PHENOBARBITAL, DIAZEPAM AND OXAZEPAM IN THE DOG.

Male beagles, 10-16 kg, were given phenobarbital (25 mg/kg), diazepam (35 mg/kg) and oxazepam (150 mg/kg) for 13 days. The drugs were administered by a stomach tube as a water suspension (particle size, less than 10-15 μ). The induction effect was studied by comparing antipyrine half-life before and two days after the dosing period. Further, the plasma concentrations of the drugs were determined on the second, seventh and twelfth day (1, 2, 4, 6, 8 and 24 h after the administration). Gas chromatographic methods were used for the assays. Behavior studies by use of T.V. and video tape were performed on the first, eight and thirteenth day of the drug treatment period.

Antipyrine half-life was found to decrease 84 and 37 % following phenobarbital and diazepam, respectively, while after treatment with oxazepam there was an increase of 22 %. Preliminary data revealed that the mean plasma concentrations (2 dogs) of the drugs at 24 h after dosing increased following all the drugs between day 2 and 7. However, the plasma concentrations decreased after 12 days administration of phenobarbital and diazepam, but were further increased following oxazepam.

Sedation and ataxia were seen after treatment with all the drugs. The degree of these behavioral symptoms changed between the three observation days and these variations seemed to be related to the induction indicated above following phenobarbital and diazepam.

K.-Fr. Sewing (Pharmakologisches Institut, D-74 Tübingen, Wilhelmstr. 56)
THE EFFECT OF PHLOXIN ON BETHANECHOL AND HISTAMINE STIMULATED GASTRIC ACID SECRETION IN RATS.

Phloxin (Na salt of tetrabromo-tetrachlorofluorescein) has been reported to inhibit histamine and pilocarpine stimulated gastric acid secretion in pylorus ligated rats (van Noordwijk, J. & P.N. Aarsen: Brit. J. Pharmacol. 1954, 9, 253 - 259) either by forming a complex with the imidazole ring of the stimulants or by inactivating endogenous histamine alleged to be released by a cholinergic stimulus. In rats anaesthetized with urethane (1.25 g/kg i.p.) prepared according to Lai (Gut 1964, 5, 327 - 341) for studying gastric acid secretion the effect of phloxin was investigated on gastric acid secretion stimulated for 3 hr by a constant infusion of bethanechol (1.1 µg/min) or histamine (10.1 µg/min). The phloxin in doses equimolar with those of the stimulants was added to the infusion fluid. Under a constant infusion of bethanechol the secretion rate rose continuously reaching a maximum after about 150 min. Phloxin did not affect the bethanechol response. With histamine a secretory plateau was built up within less than 60 min. After about 2 hr of stimulation the secretion rate dropped gradually. With phloxin the acid response was reduced by about 50 %. A sixfold increase of the phloxin dose did neither bring about an inhibition in the bethanechol experiments nor produced a greater depression of the acid response in the histamine stimulated animals. From the experiments it was concluded that bethanechol stimulates gastric secretion by itself rather than by a release of endogenous histamine which is liable to be bound and inactivated by phloxin.

C.-P. Siegers* and O. Strubelt (Institut fur Pharmakologie, Medizinische Akademie, D-2400 Lubeck, Ratzeburger Allee 160, Germany): INFLUENCE OF CONCENTRATION AND ROUTE OF ADMINISTRATION ON ETHANOL-INDUCED CHANGES OF SERUM ENZYME ACTIVITIES INDICATING LIVER INJURY.

Male rats received ethanol in a single dose of 4,8 g/kg. This dose was given either by stomach tube as a 10, 20, 40 or 60% (v/v) solution in distilled water or by intraperitoneal injection as a 10, 20 or 30% solution in saline. 2, 4, 8 and 16 hours later we determined serum enzyme activities of GOT, GPT, GLDH, and ethanol blood levels. Serum enzyme levels were found elevated only after intraperitoneal but not after oral administration of ethanol. The increase of GOT, GPT and GLDH activities in serum was highest after injection of the 30% solution and smallest after injection of the 10% solution though the applicated dose of ethanol was the same. Ethanol blood levels, on the other hand, after intraperitoneal injection of the 10% solution of ethanol were higher than after injection of the 20% or 30% solution, respectively. Intraperitoneal injection of the ethanol solutions lead to peritoneal inflammation the severity of which corresponded to the concentration of ethanol. Thus, increments of serum enzyme activities after intraperitoneal injection of ethanol seem to be a consequence rather of local irritation than of a general injury of the liver. Intraperitoneal injection, therefore, is no suitable method for investigation of the systemic hepatotoxic activity of ethanol.

S-M Aquilon [illegible] 3.
Inst. Natl. [illegible]
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CHOLINE AND A[illegible]

The distribution pattern of endogenous acetylcholine (ACh) in nerve-endings of brain indicates a two-compartment system, cytoplasm and vesicles (Whittaker et al. Biochem. J. 1964, 90, 293). Since plasma choline (Ch) is a precursor of brain ACh (Schuberth et al. J. Neurochem. 1969, 16, 695) we have studied the time-course for the appearance of labelled Ch and ACh in vesicles and cytosol of brain synaptosomes following intravenous injection of labelled Ch in mice.
After injection of 800 µCi ^3H-methyl-Ch (15 Ci/mmole) the animals were killed by decapitation and the crude mitochrondrial fraction (P2) containing the synaptosomes was isolated from a whole brain sucrose homogenate by differential centrifugation. After lysis of the P2 fraction in water the clear solution was separated on a Sephadex column into a high- and low molecular fraction. Labelled Ch and ACh were isolated by high voltage paper electrophoresis and the radioactivity measured. Endogenous ACh was bioassayed on the leech.
It was found that 1, 3 and 15 min. after injection the ratio ^3H-ACh/^3H-Ch was 5.5, 3.0 and 2.9 in the high- and 0.5 in the low molecular fraction. Control experiments with labelled ACh or Ch added to homogenates of brains from untreated mice revealed that all radioactivity (>99 %) was present in the low molecular fraction and less than 1 % in the high molecular fraction. The specific activity of the ACh was at the same time intervals 11.3, [illegible] in the low molecular frac- [illegible] nat ACh, newly synthetized in [illegible] orporated in some high mole- [illegible] endings, presumably the vesicles.

T H. Svensson[x] (Department of Pharmacology, University of Göteborg, S-400 33 Göteborg 33, Sweden): ROLE OF CENTRAL NORADRENALINE IN THE REGULATION OF MOTOR ACTIVITY AND BODY TEMPERATURE IN THE MOUSE.

Inhibition of dopamine β-hydroxylase by FLA-63 caused in the mouse decrease in motor activity and depletion of brain noradrenaline (NA). Furthermore body temperature was reduced Injection of D,L-threo-3,4-dihydroxyphenylserine (DOPS) restored behaviour, temperature and central NA. In the experiments a small dose of nialamide was given to prevent at least part of the catabolism of the NA formed from DOPS. This injection did not by itself essentially antagonize the effects of FLA-63. The effects of DOPS were likely to be mediated via the central nervous system since injection of NA, causing similar sympathomimetic and biochemical effects in the periphery as DOPS, could not antagonize the FLA-63 induced effects.

R F Squires (Research Laboratories, A/S Ferrosan, Copenhagen, Denmark) ON THE DECREASE IN CONCENTRATION OF 5 HIAA IN RAT BRAIN BY IMIPRAMINE AND RELATED SUBSTANCES.

Imipramine and related thymoleptics have been shown by various workers, using various techniques, to reduce the turnover of 5-HT in brain Two of these techniques depend on the combination with other substances (e g probenecid or 5-HT synthesis inhibitors), while a third requires the use of radioactive L-tryptophan However, DaPrada and Pletscher (J Pharm Pharmacol 1966, 18, 629) reported that imipramine alone can reduce the concentration of 5-HIAA in rat brain We have confirmed and extended these results: imipramine in doses from 3 to 25 mg/kg consistently produces highly significant decreases in rat brain 5-HIAA 3 hours after s c administration while leaving 5-HT unaffected. We also found that imipramine can partly prevent the increase in rat brain 5-HIAA induced by hyperthermia (high environmental temperature), as well as the increase in 5-HIAA after L-tryptophan loading regardless of whether imipramine is administered before or after L-tryptophan Highly significant decreases in rat brain 5-HIAA were also produced by chlorimipramine, amytriptyline, nortriptyline and chlorpheniramine Our results appear to be consistent with the hypothesis that these substances act by blocking the re-uptake of 5-HT into 5-HT nerve terminals, thus increasing the stimulation of the post synaptic 5-HT receptor and activating a little understood negative feedback mechanism which inhibits the activity of 5-HT neurones

R F Squires[x)] and J Buus Lassen (Research Laboratories, A/S Ferrosan, Copenhagen, Denmark): BIOCHEMICAL AND PHARMACOLOGICAL PROPERTIES OF p-AMINO-γ-MORPHOLINOBUTYROPHENONE (FG 5310), A NEW SELECTIVE MAO INHIBITOR

FG 5310 is the p-amino analogue of NSD 2023 (γ-morpholinobutyrophenone), another selective MAO inhibitor (R Squires and J Buus Lassen, Biochem Pharmacol 1968, 17, 369) with weak to moderate clinical antidepressant activity (Dureman et al Lakartidningen 1968, 65, 1547) FG 5310 is a more potent reserpine antagonist (ED_{50} 2-5 mg/kg) in rats and mice than NSD 2023, and has a somewhat stronger anticonvulsant activity, also of short duration FG 5310 produces selective MAO inhibition of longer duration than that produced by NSD 2023 In vivo both FG 5310 and NSD 2023 show strongest MAO inhibition with 5-HT, intermediate inhibition with tyramine, and weak inhibition with kynuramine as substrates In vitro, both NSD 2023 and FG 5310 are much weaker and less selective MAO inhibitors than they are in vivo, suggesting that active metabolites of both substances are responsible for the selective inhibition in vivo In contrast to NSD 2023, a single dose of FG 5310 does not produce a significant increase in 5-HT or a significant decrease in 5-HIAA in rat brain Combined with L-tryptophan, neither NSD 2023 nor FG 5310 produces the behavioural changes seen in mice after Nialamide or Isocarboxazide combined with L-tryptophan (jerky hyperactivity, tremor, abduction of hind legs head movements)

G Taugner, Max-Planck-Institut,Abt.Physiologie,Heidelberg Germany UPTAKE AND RELEASE OF L- AND D-ISOMERS OF NORADRENALINE BY MEMBRANES OF STORAGE VESICLES FROM THE ADRENAL MEDULLA

Catecholamine storage vesicles purified by density gradient centrifugation were shocked two times with hypo-osmotic KCl in order to separate the soluble constituents from the membranes. The catecholamine content of the membrane preparation was reduced by dialysis to 16 nmoles catecholamine/mg of membranal protein. This preparation was used for studying the stereospecifity of the uptake and the release of catecholamines. At 0°C only a low catecholamine exchange took place which was independent from ATP. Thereby the catecholamine content of the membranal protein remained constant. At 31°C in absence of ATP, catecholamine was released with similar rates wether the medium contained l- or d-noradrenaline. In presence of ATP noradrenaline was accumulated by the membranal particles. No significant differences could be detected by fluorimetric analysis between the uptake of l- and d-noradrenaline, respectively. The uptake of ^{14}C-l-noradrenaline was inhibited to 35-40% by the presence of the d-isomer. Examination of the catecholamine efflux from membranes labelled with ^{14}C-l-noradrenaline into media containing l-,d- or dl-noradrenaline gave corresponding results. The uptake and release of the stereoisomers of catecholamine by membranes of medullary storage vesicles resemble that of adrenergic nerve granules described by v.Euler and Lishajko, Acta physiol.scand. 60 217, 1964

Anja H.Tissari*) and Birgit V A Suurhasko (Department of Pharmacology, University of Helsinki, Finland) TRANSPORT OF 5HT IN SYNAPTOSOMES OF DEVELOPING RAT BRAIN.

Synaptosomes prepared from brain stem of 1-day, 1-week and 3-week-old and adult rats were incubated in Krebs bicarbonate or modified solutions stated below with 14-C-5HT (114nM) for 7 min. Concentration ratio of amine (accumulation) and accumulated plus deaminated amine (total uptake) were determined. Total uptake of 5HT in synaptosomes of 1-day, 1-week and 3-week-old rats was 49 %, 75 % and 98 % of that in the adult rat [illegible] ulation ratio of 5HT in the [illegible] d in synaptosomes of the you [illegible] 12 in the 1-day and 1-week [illegible] Incubation in a Na-free me [illegible] sucrose) abolished the total uptake and accumulation of 5HT in theadult rat synaptosomes by 85 % and 80 %, respectively, and the percentages obtained in synaptosomes of the young rats were as in the adult. When incubation was made with 1 mM ouabain or in a K-free medium after 10 min pretreatment the total uptake and accumulation of 5HT in the adult rat synaptosomes were blocked 75 % and 80 %, respectively, and the levels remaining in synaptosomes of the young rats were similar to those in the adult. 4 µM imipramine or 12 µM desipramine inhibited about 75 % of the total 5HT uptake in synaptosomes of adult and 1-week-old rats. Results suggest that already in the brain of 1-day-old rat translocation of 5HT across the neuronal membrane occurs solely by active transport.

H.J. Sørensen[x)] and N. Chakravarty (Dept. of Pharmacology, University of Odense, Denmark). POTENTIATION OF ANAPHYLACTIC HISTAMINE RELEASE FROM GUINEA PIG LUNG BY SUCCINATE AND MALEATE.

The potentiation of anaphylactic histamine release from guinea pig tissue by succinate is generally thought to be mediated through its metabolic utilization in Krebs cycle. Austen and Brocklehurst (J. exp. Med. 1961, 113, 541-557), on the other hand, consider that the succinate-induced potentiation is independent of its metabolism through Krebs cycle, since maleate - which is supposed to be metabolically inert - was also found to exert a similar effect. It is confirmed in the present work that maleate stimulates anaphylactic histamine release from minced guinea pig lung, and the stimulation is of the same order of magnitude as for succinate. The possibility that maleate may exert its stimulating effect through its metabolic utilization was considered. The present investigation shows that maleate can be metabolized to CO_2 by guinea pig lung - the rate being about 1/2 that for the convertion of succinate to CO_2. Malonate inhibits the stimulating effect of both succinate and maleate on histamine release. The findings are consistent with the assumption that both succinate and maleate enhance anaphylactic histamine release through their metabolic utilization.

K Takeya[+] and M Reiter (Institut für Pharmakologie und Toxikologie der Technischen Universität München, Biedersteiner Str. 29, Munich, Germany) EFFECT OF Mn-IONS ON ISOMETRIC TENSION AND MEMBRANE POTENTIAL OF RIGHT VENTRICULAR STRIPS OF THE RABBIT HEART.

We investigated the effect of Mn^{2+}(1-25 mM) on isometric tension and on membrane resting and action potential (MRP and AP) of muscle strips from the right ventricle of young rabbits. Mn^{2+} caused a diminution of contractile force depending on its concentration. The negative inotropic effect of 1 mM Mn^{2+} developed significantly faster at a stimulation frequency of 1/sec than at 0.1/sec, it could also be observed after longer periods of rest the first contraction 60 seconds after the addition of $MnCl_2$ to a non-stimulated muscle showed already a significant decrease in its tension. Although the contractile force of the muscle strips was almost equal in bathing solutions with normal (100 %) Na and 50 % Na, the ratio $[Ca^{2+}]/[Na^+]^2$ being kept constant, the negative inotropic effect of Mn^{2+} was markedly enhanced with 50 % Na - Mn^{2+} caused a slight increase in MRP and a diminution in overshoot Higher concentrations decreased the speed of depolarization The influence on the duration of AP was biphasically an initial prolongation, the duration of which was inversely related to the Mn^{2+}-concentration, was followed by a marked and persistent shortening of AP-duration.

(Pharmakologisches Institut der
-i Berlin 33, Deutschland):
ECHANISMUS DES AMILORID AUF DIE
PROTEINSYNTHESE.

Frühere Arbeiten haben ergeben, dass unter dem Einfluss von Amilorid der Einbau radiomarkierter Aminosäuren in Proteine des epidydimalen Fettgewebes junger Ratten gehemmt wird. Zur Klärung dieses Effektes wird im subcellulären System der Aminosäureninkorporation die Aktivität von Ribosomen, Mikrosomen und des Zellhyaloplasmas nach Einwirkung von Amilorid untersucht. Aus epidydimalem Fettgewebe, Nieren und quergestreiftem Muskel gewonnene Ribosomen inkorporieren mit Poly-Uricydylsäure als exogener Messenger-Ribonucleinsäure weniger Aminosäuren, wenn mit Amilorid in vivo vorbehandelt wird oder das Pharmakon i[illegible] -ben-
so zeigen Mikrosomen [illegible]
säuren unter Einflus[illegible] ra-
tionsleistungen. An [illegible] in
bewirkte Steigerung [illegible] ho-
ben. Die Aktivitäten der Enzyme und Faktoren des Zellhyaloplasmas, die für die Proteinsynthese benötigt werden, sind unter Amilorid-Einwirkung ebenfalls gemindert. Da sich beide Effekte, Hemmung der Syntheseleistung der Ribosomen und Hemmung der Enzyme und Faktoren des Hyaloplasmas bei der Aminosäureninkorporation, addieren, scheint der Angriff ähnlich wie bei anderen in der Literatur beschriebenen Hemmungen und Anregungen der Proteinbiosynthese nicht auf einen einzelnen Bereich der Nukleinsäure-abhängigen Proteinsynthese beschränkt zu sein.

S.O. Falkbring, P.O. Nyman, T. Wåhlstrand and P. Wistrand (Department of Biochemistry, University of Gothenburg and Department of Pharmacology, University of Uppsala, Sweden): HUMAN KIDNEY CARBONIC ANHYDRASE.

The physiological role of carbonic anhydrase in mammalian tissues has hitherto been evaluated on the assumption that the various tissues contain the same type of enzyme. However, human erythrocytes contain two carbonic anhydrase isozymes, HCA B and HCA C. They differ considerably in activity, physical and chemical properties and sensitivities towards inhibitors. To understand the action of sulfonamide diuretics it would be important to know the characteristics and distribution of carbonic anhydrase along the nephron. Donor kidneys perfused with Perfadex and free from blood were used. Homogenates from the cortex, outer and inner medulla and papillary regions were differentially centrifuged in 0.25 M sucrose at + 4°C. After centrifugation at 105.000 g x 60 min the dialysed supernatant was purified by chromatography on DEAE-cellulose. The enzyme was further purified by affinity chromatography on sulfanilamide-sephadex. The elution resulted in one peak similar to that seen for HCA C. No peak comparable to HCA B was seen. Electrophoresis on cellulose acetate strips confirmed the presence of an enzyme related to HCA C. Antibodies against the isolated human kidney carbonic anhydrase was obtained from immunized rabbits. Qualitative and quantitative immunochemical assays revealed the presence of the enzyme in the cortical medullary and papillary regions. An immunological similarity to HCA C was detected. No antigenic structure similar to HCA B was found. Thus chromatographic, electrophoretic and immunologic evidence point to the presence of an enzyme similar to the high activity erythrocyte

H.Turakka[x], (Department
maceutical
BIOPHARMACE
SOLD IN FINLAND.

Searl & Pernarowski (Can.Med.Ass.J.1967,96,1513-1520) have found considerable differences in the absorption of phenylbutazone from the different tablets sold in Canada. A comparable human study was now made from the 7 phenylbutazone preparations which are on sale in Finland (4 Finnish + 1 foreign and 1 Finnish + 1 foreign with antacids). Eight healthy volunteers (3 women and 5 men, age 25-35 years) received a tablet of 200 mg of each phenylbutazone preparation into empty stomach randomly with an interval of 14 days. Phenylbutazone content of plasma was determined at 1½, 3, 6 and 24 h after the administration according to Burns & al.(J.Pharmacol.1953, 109,346-357). The content uniformity, disintegration time (Manesty Disintegration Tester) and the rate of dissolution in simulated intestinal fluid (with standard shaking at 37°) were determined in vitro from the same lot of each product. Considerable differences between the preparations were found in the rates of dissolution and absorption in man as well as smaller differences in the maximum concentrations in plasma. A good correlation seemed to occur in the rates of dissolution and absorption. All the disintegration times filled the requirements of Ph.Nord., and did not correlate very well with the rate of absorption.

K. Turnheim*and F.Lauterbach (Institut fur Pharmakologie und Toxikologie der Ruhr-Universitat Bochum, Germany):
INTESTINAL ABSORPTION AND SECRETION OF QUATERNARY AMMONIUM COMPOUNDS

Studies in vivo by R.R.Levine & E.W.Pelikan(J.Pharmacol. exp. Ther.1961,131, 319-327)suggest that a carrier mediated mechanism may be involved in the intestinal absorption of quaternary ammonium compounds.Therefore the uptake and permeation of C-14 labeled tetraethylammonium bromide(concentration range 5o-16oo nmoles/ml) and N[1]-methylnicotinamide chloride (1-1oo nmoles/ml)were studied in vitro by use of the isolated mucosa of guinea pig jejunum (Lauterbach, F. Arch.Pharmak.exp. Path.1969,263,26-39).The uptake of the substances tested was significantly greater from the blood side than from the lumen side. This discrepancy was smallest with high concentrations and was reduced by anaerobiosis.With low concentrations of both monoquaternary compounds the permeation from the blood side of the mucosae to the lumen side was twice that in the opposite direction;this difference in flow decreased with increasing concentrations.When the mucosae were gased with N_2 instead of O_2, the flux ratio approaches 1.
These results may be explained by the existence of a secretory system for quaternary ammonium compounds in the intestinal epithelium.They support the hypothesis that the reversal of a secretory mechanism takes part in the intestinal absorption of these cationic substances (Lauterbach, F. Arch. Pharmak. 197o, 266, 388).

J.K. Seydel and G. Wassermann[x], (Borstel Research Institute and Dept. of Pharmacology Kiel, Hospitalstr. 4-6,W.-Germany):
NUCLEAR MAGNETIC RESONANCE STUDIES ON THE BINDING OF SOME BISQUATERNARY AMMONIUM COMPOUNDS TO ACETYLCHOLINESTERASE.

The specific binding of the following compounds to acetylcholinesterase (E.C. 3.1.1.7.) has been determined by means of line width studies in NMR: $R(CH_3)_2N^+-(CH_2)_6-N^+-(CH_3)_2R$, I: R = CH_3 = hexamethonium; II: R = N-propyl; III: R = phenyl-n-propyl; IV: R = phthalimido-N-propyl. The anticholinergic properties of the derivatives have been described earlier (LÜLLMANN, H. et al.: Europ. J. Pharmacol., 1969, 6, 241; GERLACH, F.D. et al.: Arzneim.-Forsch.,1970, 20, 751; LÜLLMANN, H. et al.: Biochem. Pharmacol. 1971, in press).-

The protons in the neighbourhood of the positively charged nitrogen atoms are very strongly involved in binding to the enzyme, whereas molecule groups (phenyl or phthalimide groups) located some distance from the positive center contribute to a minor degree. The influence of the substituents R on binding affinity is described and stability data are given for compound IV, where a change in NMR spectra has been observed during the measurements. The possibility of an irreversible type of binding of IV to the enzyme is discussed. - Furthermore a chemical shift has been observed and both the free and the complexed (enzyme bound) form can be studied seperately, since only a slow exchange does occur between the free and complexed form.

D. Wahl, H. Wick[+] (Departments of Biochemistry and Pharmacology, C.H.Boehringer Sohn, 6507 Ingelheim, Western Germany):
INHIBITION OF ENTERAL ABSORPTION OF SOME DRUGS BY POLYETHYLENE GLYCOL 4000.

The intestinal absorption of the quarternary drugs atropine methylbromide, hyoscine methylbromide, and hyoscine butylbromide (Buscopan®) was markedly reduced when polyethylene glycol (PEG 4000) was present, as shown by toxicity experiments in rats. This effect was not observed with the tertiary amines atropine sulfate, hyoscine hydrobromide, and with sodium pentotal. The intestinal absorption of 5 mg/kg tritium labeled hyoscine butylbromide from isolated jejunal loops in conscious rats was reduced to more than 1/2 when PEG 4000 was instilled together with the drug. PEG 4000 did not change the i.v. toxicity of hyoscine butylbromide in mice, but reduced significantly the toxicity of this drug after i.p. (mice and rats) and after s.c. injection (mice). For the three quarternary compounds studied PEG 4000 is not an inert marker. Using the PEG 4000 marker method for the evaluation of drug absorption, it is necessary to make sure first that there is no interaction between PEG 4000 and the drug to be studied.

G. Wahlström (Department of Pharmacology, University of Uppsala, Uppsala, Sweden): INTERACTION BETWEEN PROBENECID AND TWO BARBITURATES IN THE RAT.

The low lipid solubility of barbital has been used to explain the long anaesthesia induction time. Probenecid (100 mg/kg) was administered s.c. one hour prior to a barbiturate in order to see if the acid nature of barbital could be of importance.
1. Disappearance of the righting reflex after i.p. *administration of* barbital (200 mg/kg). A cross-over design with saline was used. In 23 male rats probenecid reduced the induction time by 16.7 min ($\pm$ 2.6, $P < 0.001$) from a control value of 36.3 min.
2. Dissappearance of the righting reflex after i.v. infusion of barbital (150 mg/kg in 10 min). A cross-over design with saline was used. In 22 male rats probenecid reduced the induction time with 10.4 min ($\pm$ 4.7, $P < 0.05$) from a control value of 39.9 min.
3. The threshold doses of i.v. infused hexobarbital (0.25 mg/kg/sec) needed to give a burst suppression of 1 sec or more in the EEG. In 14 male rats 1.3 mg/kg more hexobarbital was needed after probenecid ($\pm$ 1.8, $P > 0.05$) compared to an average (65.3 mg/kg) calculated on one threshold determined before and one threshold determined 1 week *after probenecid.*
Probenecid decreased the induction time with barbital. The induction with hexobarbital was not influenced. A CNS depressant effect of probenecid is thus unlikely. Since the less lipid soluble barbital penetrates the blood-brain barrier more slowly than hexobarbital the difference could be explained if the entry of barbital were delayed by a probenecid inhibited acid transport system out of the brain

G. Wahlström (Department of Pharmacology, University of Uppsala, Uppsala, Sweden): THE EFFECTS OF MELATONIN ON THE SELF-SELECTED CIRCADIAN RHYTHM OF ACTIVITY AND REST IN THE CANARY.

The self-selected rhythm of activity and rest in the canary was studied as described earlier (Wahlström: Acta Soc. Med. Upsal. 1964, 69, 241-271). The canaries were kept singly in cages which each had a separate light source. The light was extinguished as long as the birds stayed on one of the perches inside the cage. In the circadian period recorded with this method there is usually one period of activity (light) and one period of rest (darkness).
Melatonin (0.5 and 1.0 mg/kg) *was given orally as a single dose* either early (AM) or late (PM) in the activity. In each experiment the changes in the rhythm were calculated as a difference between recorded activity, rest or period length and a corresponding pre-experimental average based on 5 circadian periods.
The following average changes were recorded in the period in which melatonin was given (S.E.M.):

	Melatonin 0.5 mg/kg		Melatonin 1.0 mg/kg	
	AM (N=11)	PM (N=10)	AM (N=10)	PM (N=9)
Rest	0.91(0.39)	1.01(0.56)	1.68(0.49)	1.05(0.32)
Period length	0.03(0.11)	-0.15(0.08)	0.04(0.06)	-0.30(0.09)

In the AM experiments this increased rest (and a correspondingly decreased activity) was due to a "nap" within the activity period in 4 experiments out of 12 with 0.5 mg/kg *and in 7 experiments out of* 10 with 1.0 mg/kg. In the PM experiments the increased rest depended mainly on a earlier roosting. Melatonin thus can induce the canary to choose an enviroment which usually is connected wi[illegible] *of 19 experiments* with 1.0 mg/kg this had occurred wi[illegible] after melatonin.

J.K. Seydel and O. Wassermann[x], (Borstel Research Institute and Dept. of Pharmacology Kiel, Hospitalstr. 4-6,W.-Germany):
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S.-M. Aquilonius and B. Winbladh[x] (Research Institute of National Defence, Sundbyberg, Sweden): CEREBROSPINAL FLUID CLEARANCE OF CHOLINE.

Several types of substances including some quaternary amines are cleared out of cerebrospinal fluid (CSF) partly by an active transport mechanism (Schanker, L.S. *et al.* Life Sci. *1962*, 10, *515-521*). Most of these substances and also choline (Ch) are taken up in choroid plexa *in vitro* with active transport (Tochino, Y. & Schanker, L.S. Amer. J. Physiol. *1965*, 208, *666-673*, Winbladh, B., unpublished observations). Thus, the mode of exit out of CSF *for Ch was* investigated with ventriculo-cisternal perfusion in anaesthetized dogs. ^{3}H-Ch was used and CSF bulk flow was determined with ^{14}C-inulin. Artificial CSF was infused in a lateral ventricle (145 µl/min) and the effluent collected from a cannula in cisterna magna. The Ch clearance not attributable to bulk flow was $1.1 \cdot 10^{-9}$ mmoles/min at an infusion concentration of 10^{-8} M, $5.7 \cdot 10^{-7}$ mmoles/min at $7 \cdot 10^{-6}$ M and $5.5 \cdot 10^{-6}$ mmoles/min at 10^{-4} M. At these concentrations bulk flow was responsible for 28 %, 41 % and 63 % of total Ch clearance respectively. The clearance mechanism thus appears to be concentration dependant, and cannot solely be due to simple diffusion. At physiological ventricular Ch concentration ($5 \cdot 10^{-6}$ M) and a physiological CSF flow bulk clearance would probably be less than 40 per cent. This limits the possibility to evaluate ventricular CSF Ch concentration from lumbar CSF (Cf. Aquilonius *et al.*, this meeting).

H. Winkler*, J.A.L. Schöpf, H. Hörtnagl and Heide Hörtnagl (Dep. of Pharmacology, University of Innsbruck, Austria):
SUBCELLULAR DISTRIBUTION OF NEWLY SYNTHESISED CATECHOLAMINES, ATP AND CHROMOGRANINS IN BOVINE ADRENAL MEDULLA.

Bovine adrenal glands were perfused retrogradely with Tyrode solution. One hour after the start of the perfusion ^{3}H-leucin, ^{3}H-tyrosine, ^{32}P-phosphate or $^{45}CaCl_2$ were injected into the perfusion medium. 3 min. or 4 hrs. later the subcellular fractions of the adrenal medullae were isolated. Already three minutes after the injection of ^{3}H-tyrosine the bulk of the labelled catecholamines was found in chromaffin granules. Three minutes after ^{32}P most of the labelled ATP was present in the mitochondrial fraction whereas chromaffin granules contained a relatively small amount. Four hours after ^{32}P this pattern was reversed since chromaffin granules now contained most of the labelled ATP. Similar results were obtained with ^{45}Ca. Three minutes after ^{3}H-leucin injection labelled chromogranins, which are the soluble proteins of chromaffin granules, were present in the microsomal fraction. Four hours after ^{3}H-leucin the labelled chromogranins appeared in particles which apparently represent newly formed chromaffin granules. The membrane proteins of these cell particles were not labelled.
These results indicate that the chromogranins are synthesised in the endoplasmic reticulum and are then transferred, probably via the Golgi region, into chromaffin granules. ATP, calcium and catecholamines are incorporated into chromaffin granules which are already segregated from the Golgi apparatus.

G.Zetler* and O.Strubelt (Institut für Pharmakologie, Medizinische Akademie, D-2400 Lubeck, Germany). ACTIONS OF ANTIARRHYTHMIC DRUGS ON REFRACTORY PERIOD AND CONTRACTILITY OF ISOLATED RAT AND GUINEA-PIG ATRIA.

It is not known whether the hearts of rat and guinea-pig have the same sensitivity for the negative actions of antiarrhythmic drugs on *refractory* period and contractility. We proved this question *in electrically* driven isolated left atria. By dosing the drugs cumulatively the following equipotent concentrations (mM) were obtained.

	Refractory period. Prolongation by 50 msec		*Strength of contraction* Reduction by 50%	
Drug	Rat	Guinea-pig	Rat	Guinea-pig
Quinidine	0.17	0.65	0.16	0.19
Ajmaline	0.05	0.015	0.13	0.027
Sparteine	0.81	0.48	3.29	>4.5
Phenytoin	1.79	2.74	0.96	0.75
Procainamide	4.92	1.78	8.44	>11.0
Lidocaine	0.23	0.13	0.22	0.22
Brufacaine	0.89	0.51	0.89	0.43
Propranolol	0.053	0.042	0.041	0.014
Iproveratril	0.0036	0.012	0.0025	0.0037

In both objects, ajmaline, propranolol, and iproveratril were most active, and the therapeutic index was highest for sparteine and lowest for phenytoin. We consider the isolated left rat atrium useful for work on antiarrhythmic drugs.

A. Ziegler (Department of Pharmacology, Hospitalstr. 4-6, 23 Kiel, W.Germany): THE FREQUENCY OF ASSOCIATION AS DETERMINING FACTOR FOR THE TIME COURSE AND THE MAGNITUDE OF THE RESPONSE INDUCED BY CHOLINOMIMETIC DRUGS.

The time course of the mechanical response of isolated, electrically driven guinea pig's atria elicitated by different cholinomimetic drugs has been determined (BIEGER et al., 1970). Under identical experimental conditions the uptake of some labelled *cholinomimetic* compounds has now been measured. The time course of drug uptake was, however, not correlated to the time course of the response. Assuming that the frequency of association (k_{12}·[A]·[R_f]) is determining for the magnitude of the response, a good correlation was found between the time course of the frequency of association and that of the mechanical effect. - If a certain frequency of association is necessary to cause a given effect, the product k_{12}·[A]·[R_f] demonstrates, that 1) a drug with a high association rate constant (k_{12}) needs a lower concentration [A] to evoke a particular response than a compound with a low k_{12}, and 2) that a drug with a low dissociation rate constant (k_{12}) reduces the number of free binding sites (R_f), thus *producing* a "*fading*". - The predictions derived from our kinetic approach have been checked experimentally and could be verified. The frequency of association determines the time course and the magnitude of the response, a fading phenomenon can only be observed, if a considerable fraction of all receptors is involved; the dissociation constant K is not identical with the ED_{50} of the drugs investigated.

J. Doss and P.A. van Zwieten+ (Department of Pharmacology, Hospitalstr. 4-6, 23 Kiel, W.Germany): THE CHARACTER OF THE CHOLINERGIC RECEPTORS THAT ARE INVOLVED IN THE PRODUCTION OF GASTRIC ACID.

The effects of various cholinergic drugs with muscarine or nicotine like activity on gastric acid production was studied in the anaesthetized rat. The stomach was perfused continuously in situ and the pH of the perfusate was recorded. For the increase in acid production the following sequence of activity was observed:
Arecaidine-ethyl-ester-methiode ≈ arecaidine-methyl-ester-methiodide ≈ ACh ≈ DFP > neostigmine > tremorine ≈ pilocarpine ≈ carbachol > acetyl-β-methylcholine > arecaidine-ethyl-ester (tert. form) > muscarine. Arecoline was not effective.
This sequence is entirely different from that reflecting the muscarinic action on heart, ileum, blood pressure or salivation. Ganglionic stimulants (DMPP, nicotine) or ganglionic blocking agents did not influence acid production. The results suggest that for the cholinergic receptors involved in gastric acid production the classical concept of muscarine or nicotine sensitive receptors cannot hold true.
Therefore, the existence of a new type of cholinergic receptors in the stomach is postulated.

ABSTRACTS OF

INVITED LECTURES AND

SYMPOSIA

H.Schildknecht[x)] (Institute of Organic Chemistry, University of Heidelberg, Germany) CHEMICAL WARFARE AMONG INSECTS.

In the struggle for existance of many insects pure chemistry is of decisive importance. With chemical substances they create and control their living space They survive through poison' They have reached evolutionary peaks in the development of their defense mechanism through substances produced for protection. Thus the highly toxic quinones will be produced only at the first sign of danger in the bombardier beetle. In addition they will not merely be secreted as with the Tenebrionids but shot forth explosively as a hot toxic cloud in the direction of the attacker. (Schildknecht, H Angew. Chem. 1957, 69, 62). No such explosion would be conceivable in any other type of biochemical system except the bombardier beetle's. - Another evolutionary peak is reached in the defensive chemistry of the water beetles, they are extremely well adapted to living in water. Their pygidial defensive glands contain phenols with which they can protect themselves against large enemies like pike, trout, frogs or even small mammals. (Schildknecht, H. Angew Chem. 1970, 82, 17-25). - The most successful insects are ants which is, for example, proven by their feeding habits. The leafcutting ants are highly developed because they are not dependent on finding food by accident they grow their own fungus garden. To cultivate their fungus garden they use growth hormones specially produced for that purpose in their metathoracic glands (Schildknecht, H. & K.Koob Angew. Chem. 1971, 83, 110, Intern. Ed. 1971, 10, 124-125).

Wm. B. Deichmann* (Dept. of Pharmacology and Res. and Teaching Ctr. of Toxicology; University of Miami School of Med., P.O. Box 8216, Coral Gables, Florida 33124): THE DEBATE ON DDT.

DDT must be given credit as the single most effective agent in the prevention of illness and death caused by insects transmitting malaria, typhus, yellow fever, river blindness, and other vector-borne diseases. Equally, DDT has played a leading role in our fight against starvation. Misuse and overuse has resulted in pollution of soils, waters, and in elevated tissue concentrations in fish, birds, and plants. The quantities ingested by the general population who buy foods at markets handling U.S. FDA-controlled products, present no hazard to health. DDT in tissues of beagles in conc. comparable to the elevated conc. in certain healthy pesticide operators and human volunteers produced delayed estrus, reduction of libido, stillbirths, reduced milk production and mammary development, and high mortality in the offspring. In dogs, small amounts of aldrin, when added to the daily DDT intake, caused a prompt rise in DDT, DDE, and DDD in blood and abdominal fat. In Osborne-Mendel strain rats fed a diet supplemented with DDT and/or aldrin, six days of starvation, while inducing marked loss of a) body weight, b) total body lipids, and c) pesticides, induced a significant increase in the concentration of pesticides in the remaining body fat. In addition, in female rats, starvation resulted in an increase in concentration, and in an increased retention of total DDT (and dieldrin) in the liver, brain, and kidney. In male rats, loss of pesticides from the body was associated with reduced tissue levels and decreased total retention of pesticide per organ. Starvation effects were most marked in weanling, least marked in old rats. DDT, while stored in fat, is not in dead storage.

M. Berlin (Institutionen för Hygien, Lunds universitet, Lund, Sweden):
THE MENACE OF MERCURY COMPOUNDS.

Different mercury compounds are absorbed, retained, and excreted by mammals differently. The chemical form that mercury is absorbed in determines its distribution and turnover in the tissues and its toxic effect. Water-soluble mercury compounds are alkylated to methyl mercury (MeHg) in aquatic milieu. Mammals have a limited ability to excrete MeHg or to break its carbon-mercury bond, and it accumulates in tissues even at low intake, especially in CNS. Experiments on Saimiri sciureus have shown that MeHg accumulates subcortically in the cerebellum and cerebrum, especially in the visual cortex, and causes atrophia of the cerebral cortex with vacuolization, degeneration and loss of neurons. The accumulation occurs slowly and reaches a maximum about a month after initial absorption.
The biological halflife of MeHg in man is 70 days, which means a daily excretion of about 1% of the body burden. About 10% of the body burden is found in the brain, where a concentration of about 5 μg/g gives rise to toxic, morphological changes that can be lethal. At equilibrium, blood concentration of mercury reflects brain concentration. Neurological signs have been observed at blood concentrations of MeHg around 0,2 μg/g whole blood, which corresponds to a daily intake of 4 μg/kg bwt/day based on epidemiological investigations, and embryotoxic effects on CNS have been observed at exposure levels below those causing clinical signs in adults. At concentrations lower than 0,2 μg/g whole blood, increased chromosome breakage in lymphocytes has been found in humans who have consumed contaminated fish. Swedish experts have decided that the acceptable daily intake of MeHg in man ould not exceed 0,4 μg/kg bwt/day. The average intake at present in the Swedish population not exposed to contaminated fish is about 0,1 μg/kg bwt/day or one fish meal per week.

SYMPOSIUM I

Intestinal absorption

D. H. Smyth. (Department of Physiology, University of Sheffield, England). INTRODUCTION.

In considering transfer mechanisms by the intestine much use is made of analogies with enzymology. The carrier is thought to contain an active site similar to the active centre of enzymes, and on the basis of this Michaelis-Menten kinetics are applied to intestinal transfer and the effects of competition or inhibition are described in these terms. In addition to recognising similarities between carriers and enzymes it is important to recognise differences, and some account will be given of the general resemblances and differences between enzymes and carrier mechanisms in intestinal transfer.

In enzymology it is assumed that the rate-limiting stage in the process is the attachment of the enzyme with the substrate and hence the rate of the process is proportional to the saturation of the carrier. Carrier processes are more complex and while attachment to . e carrier is one stage it may or may not be the rate-limiting stage. Hence while the kinetic constants give information about the overall rate of transfer this is not necessarily attachment of absorbate to the carrier.

P F. MILLINGTON (BioEngineering Unit, University of Strathclyde, Glasgow, Scotland) ANATOMY OF THE SMALL INTESTINE.

In the two decades since the introduction of commercial electron microscopes, details of the structure and spatial distribution of intestinal cells, organelles and many enzymes have been elucidated Interest now appears to centre on development and growth and various functional aspects of the organ. One of the methods found to be extremely useful has been tissue fractionation. Using this basic approach brush borders have been isolated and studied in some depth (Millington, P F et al, J Cell Bio 14, 125, 1962, J Cell Sci 1, 415, 1966, Z Zellforsch 87, 401, 1968, Life Sciences 7, 839, 1968, Histochem J., 1, 311, 1969). Scanning electron microscopy has and will reveal features of the epithelial cells not readily shown by earl er methods (Millington, P.F. et al, J. Microscopy 89, 339, 1969) X-ray analys s of tissue pieces and sections will help us to locate accurately chemical elements in the tissue. These microscopical techniques together with methods for rap d quantitat ve assessment now being developed form the current tools for research.

In developmental terms, the final structural pattern of the small intestine is achieved in mammals shortly after birth. While the embryo is in utero little investigation can be done without considerable disturbance to the mother. The early post-natal period during which changes are still continuing is, therefore, of considerable interest. Studies on the structural changes in the villi, of cell migration, of the ep thelial cells and their microvilli will be described. Effects of amino-acetyl nitrile bisulphate on collagen in the submucosa and villus core indicate that the mucosal structure is not s mply related to the amount of collagen present although there is a decrease in mechanical strength with a decrease and change in collagen. Mitoses and hence cell turnover during this period also change, the pattern of change in the ileum being different to that in the duodenum. Cell turnover is to some extent under the influence of cortisone. An injection before the fifth day after birth in rats appeared to increase the duodenal mitotic rate whereas if given on or about the eighth day a depression in rate was found. No significant effects were found in the ileum Cortisone also influences phosphatase activity (Moog, F. Dev Biol 3, 153, 1961) During our studies on the apical region of cells from a number of d fferent species we have come across a curious feature which has only been seen during a developmental phase, never in the adult A pattern of grooves and ridges has been found which form a communicating system at the base of the microvilli. Speculation on its significance is at present all that is possible.

D.Winne (Pharmakologisches Institut der Universitat, Wilhelmstrasse 56, D-7400 Tübingen, BRD):
BLOOD FLOW AND THE KINETICS OF INTESTINAL DRUG ABSORPTION.
Jejunal loops in anaesthetized rats (urethane) were perfused with 14-C-labelled substances in *buffered* saline solutions at pH 7 (exceptions see below); concentration 20 to 746 µM, tritiated water 11 µCi/ml. The blood flow, the disappearance rate from the intestinal lumen and the appearance rate in the intestinal venous blood were determined simultaneously. The disappearance rate was greater (especially at low blood *flow values*) than or about equal to the appearance rate; the difference of these two rates is the amount of substance *transferred* to the serosal side of the gut. At intermediate blood flow (1 ml/min/g wet tissue weight) and for a concentration of 50 µM in the perfusion solution the appearance rate lies between 5 and 0.05 nMol/min/g. In general, a decrease of blood flow (from about 2 *to 0.2 ml/min/g*) diminishes and an increase of the blood flow increases both absorption rates. The dependence on blood flow is greater for the appearance than for the disappearance rate. Both absorption rates and their dependence on blood flow decrease in the following order: tritiated water; aniline (at pH 8), benzoic acid (at pH 6.2), salicylic acid (at pH 6), antipyrine (at pH 8); ethanol, methanol, amidopyrine, glycerol, ethylene glykol; urea, erythritol; ribitol, mannitol. The appearance rate of tritiated water is strictly dependent on blood flow: its absorption is blood flow limited. The absorption of ribitol and mannitol is independent of blood flow. For details see: Ochsenfahrt, H., & D.Winne: Naunyn-Schmiedebergs Arch. Pharmak. 1969, 264, 55-75; Winne, D., & J.Remischovsky: Naunyn-Schmiedebergs Arch. Pharmak. 1971, 268, 392-416, and 1971, in press.
By means of a modified pharmacokinetic four-compartment-model (intestinal lumen, subepithelial intestinal wall, flowing blood in the capillary net distributed homogeneously in the subepithelial intestinal wall, serosal bath) equations can be derived for the dependence of the absorption rates on blood flow (Winne, D. : Naunyn-Schmiedebergs Arch. Pharmak. 1971, 268, 417-433). The theoretical curves calculated by the equations well fit the experimental data (Winne, D. : Med. Welt 1971, 22, 632-640). A simplified equation facilitates the theoretical interpretation of the results. The resistance of the tissue between the intestinal lumen and the blood can be divided into two parts: R-1, the resistance of the tissue between the intestinal lumen and the capillary wall (mainly the resistance of the epithelium), and R-2, the resistance of the drainage system (including the blood flow). A high permeability of the epithelium means a small resistance R-1, the resistance R-2 determines the absorption: The appearance rate is blood flow limited. A low permeability of the epithelium means a large resistance R-1 and the resistance R-2 can be neglected: The absorption rates are independent of blood flow. With decreasing permeability of the epithelium the dependence of the absorption on blood flow decreases from the blood flow limited absorption *of* tritiated water to the blood flow independent absorption of ribitol and mannitol.

P.F. MILLINGTON (BioEngineering Unit, University of Strathclyde, Glasgow, Scotland). ANATOMY OF THE SMALL INTESTINE.

In the two decades since the introduction of commercial electron microscopes, details of the structure and spatial distribution of intestinal cells, organelles and many enzymes have been elucidated. Interest now appears to centre on development and growth and various functional aspects of the organ. One of the methods found to be extremely useful has been tissue fractionation. Using this basic approach brush borders have been isolated and studied in some depth (Millington, P.F. et al; J.Cell Bio. 14, 125, 1962; J. Cell Sci. 1, 415, 1966; Z. Zellforsch 87, 401, 1968; Life Sciences 7, 839, 1968; Histochem. J., 1, 311, 1969). Scanning electron microscopy has and will reveal features of the epithelial cells not readily shown by earlier methods (Millington, P.F. et al; J. Microscopy 89, 339, 1969). X-ray analysis of tissue pieces and sections will help us to locate accurately chemical elements in the tissue. These microscopical techniques together with methods for rapid quantitative assessment now being developed form the current tools for research.

In developmental terms, the final structural pattern of the small intestine is achieved in mammals shortly after birth. While the embryo is in utero little investigation can be done without considerable disturbance to the mother. The early post-natal period during which changes are still continuing is, therefore, of considerable interest. Studies on the structural changes in the villi, of cell migration, of the epithelial cells and their microvilli will be described. Effects of amino-acetyl nitrile bisulphate on collagen in the submucosa and villus core indicate that the mucosal structure is not simply related to the amount of collagen present although there is a decrease in mechanical strength with a decrease and change in collagen. Mitoses and hence cell turnover during this period also change; the pattern of change in the ileum being different to that in the duodenum. Cell turnover is to some extent under the influence of cortisone. An injection before the fifth day after birth in rats appeared to increase the duodenal mitotic rate whereas if given on or about the eighth day a depression in rate was found. No significant effects were found in the ileum. Cortisone also influences phosphatase activity (Moog, F. Dev. Biol. 3, 153, 1961). During our studies on the apical region of cells from a number of different species we have come across a curious feature which has only been seen during a developmental phase, never in the adult. A pattern of grooves and ridges has been found which form a communicating system at the base of the microvilli. Speculation on its significance is at present all that is possible.

D.Winne (Pharmakologisches Institut der Universität, Wilhelmstrasse 56, D-7400 Tübingen, BRD):
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Jejunal loops in anaesthetized rats (urethane) were perfused with 14-C-labelled substances in buffered saline solutions at pH 7 (exceptions see below); concentration 20 to 746 µM, tritiated water 11 µCi/ml. The blood flow, the disappearance rate from the intestinal lumen and the appearance rate in the intestinal venous blood were determined simultaneously. The disappearance rate was greater (especially at low blood flow values) than or about equal to the appearance rate; the difference of these two rates is the amount of substance transferred to the serosal side of the gut. At intermediate blood flow (1 ml/min/g wet tissue weight) and for a concentration of 50 µM in the perfusion solution the appearance rate lies between 5 and 0.05 nMol/min/g. In general, a decrease of blood flow (from about 2 to 0.2 ml/min/g) diminishes and an increase of the blood flow increases both absorption rates. The dependence on blood flow is greater for the appearance than for the disappearance rate. Both absorption rates and their dependence on blood flow decrease in the following order: tritiated water; aniline (at pH 8), benzoic acid (at pH 6.2), salicylic acid (at pH 6), antipyrine (at pH 8); ethanol, methanol, amidopyrine, glycerol, ethylene glykol; urea, erythritol; ribitol, mannitol. The appearance rate of tritiated water is strictly dependent on blood flow: its absorption is blood flow limited. The absorption of ribitol and mannitol is independent of blood flow. For details see: Ochsenfahrt, H., & D.Winne: Naunyn-Schmiedebergs Arch. Pharmak. 1969, 264, 55-75; Winne, D., & J.Remischovsky: Naunyn-Schmiedebergs Arch. Pharmak. 1971, 268, 392-416, and 1971, in press.
By means of a modified pharmacokinetic four-compartment-model (intestinal lumen, subepithelial intestinal wall, flowing blood in the capillary net distributed homogeneously in the subepithelial intestinal wall, serosal bath) equations can be derived for the dependence of the absorption rates on blood flow (Winne, D. : Naunyn-Schmiedebergs Arch. Pharmak. 1971, 268, 417-433). The theoretical curves calculated by the equations well fit the experimental data (Winne, D. : Med. Welt 1971, 22, 632-640). A simplified equation facilitates the theoretical interpretation of the results. The resistance of the tissue between the intestinal lumen and the blood can be divided into two parts: R-1, the resistance of the tissue between the intestinal lumen and the capillary wall (mainly the resistance of the epithelium), and R-2, the resistance of the drainage system (including the blood flow). A high permeability of the epithelium means a small resistance R-1, the resistance R-2 determines the absorption: The appearance rate is blood flow limited. A low permeability of the epithelium means a large resistance R-1 and the resistance R-2 can be neglected: The absorption rates are independent of blood flow. With decreasing permeability of the epithelium the dependence of the absorption on blood flow decreases from the blood flow limited absorption of tritiated water to the blood flow independent absorption of ribitol and mannitol.

B.G. Munck (Institute of Medical Physiology, Dept. A, University of Copenhagen, Juliane Mariesvej 28, 2loo Copenhagen Ø, Denmark):
METHODS FOR INVESTIGATION OF INTESTINAL ABSORPTION.

The methodology of investigation of the absorptive function of the small intestine will be discussed on the assumption that the aims of the studies are to elucidate the roles played in absorption by the bordering membranes of the epithelial cells, the brush border membrane and the serosal membrane. These aims can not be reached by IN VIVO techniques as these do not allow a sufficiently good control of the conditions under which the epithelium functions. Also the isolated epithelial cell is unsuitable as in this preparation the functional polarization is eliminated. If the IN VITRO intestinal preparation is considered as a three compartment system (Munck & Schultz: J. Gen. Physiol. 1969, 53, 157-182) it is realized that in order to describe its function one needs at least information of steady state intraepithelial accumulation, steady state influx across the brush border, steady state transmural unidirectional fluxes in both directions as functions of the concentration of the substance the absorption of which is studied. By means of examples it will be shown that all three types of information may be needed to interprete on the cellular level effects resulting from experimental changes in the conditions under which the epithelium functions. It will be demonstrated that the function of the nonepithelial tissues necessitates that these are excluded from the preparation used to estimate the ability of the epithelium to accumulate transported substances.
Data of the effect of an amino acid on transmural fluxes of sodium and chloride will be discussed which indicate that the function of in vitro preparations can be modified by the nonepithelial tissues not only because these act as a diffusion barrier to the transported substances but also, may be, because they trap functionally active metabolites in the vicinity of the epithelial cells which in vivo are carried away by the blood stream.

E Skadhauge (Institute of Medical Physiology, Dept. A, Juliane Mariesvej 28, 21oo København Ø, Denmark):
INTESTINAL ABSORPTION OF SALT AND WATER

The purpose of this communication is to review briefly the characteristics of the Na and water transport across the wall of the small and large intestine, and the mechanisms involved in the coupling of NaCl and water flow The intestinal Na^+ absorption works against an electrochemical gradient and may thus be considered active The transport depends upon the luminal Na concentration and displays saturation kinetics In the small intestine the transmural electric potential difference is small and the Na absorption is stimulated by the presence of glucose and amino acids The colon Na absorption differs in being associated with an electric potential difference of 4o-6o mV lumen negative, and in being influenced by mineralo-corticoid hormones and not augmented by the presence of non electrolytes In the lower small intestine the Na and Cl concentrations are close to those of plasma, and the absorbate is isosmotic to plasma, whereas in colon the Na pump absorbs most of the Na and reduces the faeces Na concentration to approximately 2o meq/l The absorbate is hyperosmotic to plasma
According to the classical concept the water flow between the intestinal lumen and plasma is passive and proportional to the transmural osmolality difference Several lines of evidence have demonstrated that also other factors influence the net water flow First and foremost in experiments on dog, cat, and rat small intestine, chicken colon, and eel intestine water absorption has been observed against an osmotic difference of around 1oo mOs Second, even in the cases in which the luminal osmolalities were lower than those of plasma the osmotic permeability coefficients measured for the intestinal wall were too small to account for the actual water flow observed Third, water absorption from isosmotic luminal fluids would only take place if Na was absorbed This water flow has in rat small intestine and in human rectum been observed to be proportional to the Na absorption rate Furthermore the author observed on eel intestine that the maximal osmolality difference against which the intestine could transport water was proportional to the transmural net NaCl flow These findings suggest a causal relationship between the apparently non-osmotic water flow and net NaCl flow P F Curran suggested that the Na flow created a local hypertonic area in or between the cells This would allow water to move osmotically into this region, and if the wall of the region on the serosal side did not restrict solute flow, a small hydrostatic force would suffice to move water from the region into interstitial fluid on the serosal side without creating an adverse osmotic force J M Diamond has provided evidence from rabbit gall bladder that the actual site of the hyperosmotic area may be the lateral intercellular spaces All the findings quoted above are readily explained by this hypothesis They thus support the concept that the apparently non-osmotic water flow is osmotic with the salt flow as driving force

B. Beermann, K. Hellström and A. Rosén[x] (Clinical Pharmacology Laboratory and Department of Medicine, Serafimerlasarettet, 112 83 Stockholm, Sweden): INTESTINAL ABSORPTION OF DRUGS IN MAN.

In spite of the extensive medical use of many oral drugs very little is known about their gastrointestinal absorption in man. We have adopted a radiochemical technique to study this transport for different drugs.

Water solutions of ^{3}H or ^{14}C labeled drugs together with a nonabsorbable marker were administered orally or intraintestinally to healthy subjects provided with a gastrointestinal tube. The site and the degree of absorption of radioactivity were determined by comparing the radioactivity per unit nonabsorbable marker of the aspirates at different gastrointestinal levels and that of the test solution. The technique of calculating the absorption is only accurate under certain conditions e.g. the drug should not undergo decomposition and the label must remain attached to the drug within the investigated part of the gastrointestinal tract.

The initial and maximal plasma levels of label were often correlated to the site and the degree of absorption.

A high percentage yield of radioactivity in the urine after an instillation of labeled compounds to a more distal part of the small intestine indicated a substantial absorption in lower segments of the intestinal tract.

The drugs studied so far include quaternary ammonium compounds, a tertiary amine and cardiac glycosides. They demonstrate all degrees of uptake as well as differences in the site of absorption.

Certain drugs may undergo decomposition before being absorbed. An example of such a compound was encountered in the investigation.

J Aguiar (Pharmaceutical research and development Pfizer nc., Groton, Connecticut o6340, USA)· PHYSICAL AND PHARMA-EUTICAL FACTORS AFFECTING DRUG ABSORPTION.

he availability and absorption of a drug from orally in-ested dosage forms are dependent on a number of physical roperties of the drug such as size and morphology of drug articles, its crystalline state, wettability, solubility nd rate of solution. These physical properties can influ-nce the build up of an effective concentration of the drug t the site of absorption, particularly when a drug has a elatively low solubility. In addition the manufacturing rocesses and the vehicular components utilized in formu-ating a drug into a dosage form can also affect its avail-bility for absorption. The theoretical aspects involving hese physical properties of drugs are discussed and their pplicability or nonapplicability is demonstrated using elected examples sifted from the literature. Similarly the nfluence of pharmaceutical manipulations is discussed, some f the less common and less appreciated influences of the hysical and pharmaceutical factors on drug availability re emphasized.

W.FORTH (Inst.f.Pharmakol.& Toxikol. Homburg/Saar,W.-Germany)

FACTORS INFLUENCING THE ABSORPTION OF HEAVY METALS

In order to limit the scope of this paper all factors which influence the availability of heavy metals for absorption in the gut lumen have to be set aside. These are for instance the foodstuffs and the H^+-concentration of the digestive excretions in the gastro-intestinal tract. This paper deals exclusively with the transfer of heavy metals across the mucosal cells of the intestine, i.e. with the very process of absorption.

For an understanding of the absorption process of biological significant heavy metals such as Cu, Zn or Fe, the fact that in the mucosal cells do exist transfer systems proved to be important. The transfer of Fe for instance which depends on cellular metabolism, takes place preferentially from the mucosal- to the serosal side. The ability of the mucosal epithelium to transfer Fe in this direction is increased in iron deficiency. The transfer system for Fe has regulative functions for absorption in as much as it warrants the optimal absorptive utilization of Fe taken up with foodstuffs (FORTH et al., Naunyn-Schmiedebergs Arch. Pharmak. 252, 224 (1965); 260, 50 (1968); 261, 434 (1968).)

Fe is bound in and transfered across the mucosal epithelium on one hand with a remarkable degree of specificity. On the other hand, however, the transfer system does react with heavy metals chemically related to Fe as for instance with Co and Ni, with Mn and even with Zn, but not with Cu. (FORTH et al., Med. Pharmacol. exp. 15, 179 (1966); FORTH, W. in: Trace element metabolism in animals (Mills, Ed.) p. 298, Livingstone 1970.) This fact has two consequences of practical significance. Firstly, heavy metals can mutually inhibit one another in the process of absorption. Secondly, also metals without biological significance and even toxic metals can be incorporated into the organism by means of a transfer system for biometals. In addition it can be stated that similar results as with Fe have been obtained with Cu and Zn.

In the food heavy metals do not exist as metal ions, but due to their ability to form coordinate bonds, as metal complexes. When the effective stability of the metal complexes is low they dispose their metals to the binding sites of the transfer systems. This holds for most complexes formed by metals with ligands of the food. When the effective stability is high the physicochemical properties of the compounds are determining: in contrast to polar and highly water soluble complexes sparely polar and lipid soluble compounds are well absorbed. (FORTH et al., Naunyn-Schmiedebergs Arch. Pharmak. 252, 242 (1965); 261, 314 (1968).)

H Keberle*), (Chemical Research Laboratories, Pharmaceutical Division, CIBA GEIGY Limited, Basle/Switzerland) ABSORPTION OF SOME ANTIBIOTICS

The first part of the paper deals with the chief problems which it is hoped may be elucidated by pharmacokinetic studies of enteral absorption in man The various experimental methods currently employed in the investigation of these problems are critically discussed and some special difficulties pointed out A new and efficient technique, known as the "method of internal reference", is described, and an example of its application in the evaluation of the antibiotic rifampicin is given

In the second part, two special problems are discussed, in connection with which absorption studies can be of particular importance

1 The influence of enterohepatic circulation and uptake by the liver on the blood concentrations of antibiotics, as exemplified by the pharmacodynamics of various rifamycins

2 The significance of the chemical and biochemical, i e enzymatic, stability of the cephalosporin antibiotics with regard to their absorption and concentration in the blood

F. Lauterbach (Institut fur Pharmakologie und Toxikologie der Ruhr-Universitat, Bochum, Im Lottental, Germany)

ABSORPTION OF CARDIAC GLYCOSIDES

The intestinal absorption of cardiac glycosides shows some peculiarities which cannot be explained by a diffusion of these substances(for references see Lauterbach, F. Arch. Pharmak. exp. Path. 1969, 263, 26-39). For further investigations a suitable in vitro preparation was developped. It uses the isolated mucosal epithelium of guinea pig jejunum as separating membrane(0.2 sqcm)between two chambers (filled with 0.2 ml solution each). So far uptake from and penetration in both directions of four tritium-labeled glycosides (digitoxin, digoxin, convallatoxol, ouabain) have been studied and compared with the behaviour of other substances. - Relative uptake (cellular concentration as percentage of concentration offered) of cortisol and phenobarbital is independent of the applied concentration and the application side. Contrary to this, the relative uptake of digoxin and convallatoxol decreases within certain ranges of increasing concentration. Uptake from blood side is always higher than from lumen side. Anaerobiosis or addition of 2,4-DNP increases uptake from the lumen and decreases uptake from the blood side significantly ($p < 0.0027$) (tab). Incubation at 2° reduces relative uptake to 6% and eliminates the differences in uptake between the two glycosides, application sides and different concentrations.- For cortisol and phenobarbital the penetration rates in both directions are identical. On the other hand digoxin and convallatoxol penetrate up to 14 times faster in the direction from the blood to the lumen than vice versa. Under anaerobic conditions or after addition of 2,4-DNP the flux ratio is 1. Experiments in vivo substantiated these results. After i.v. application guinea pigs as well as rats secrete both glycosides into the intestinal lumen, concentration gradients up to 25 fold (guinea pig,digoxin) were established.-From these and further results the following conclusions can be drawn Transport mechanisms for cardiac glycosides are located in the basal and/or lateral membrane as well as in the luminal membrane of the mucosal cell. The basal mechanism pumps digoxin and convallatoxol into and the luminal mechanism out of the cell. The absorption of cardiac glycosides then can be explained as a superposition of diffusion and transport processes, the latter causing absorption by the reversal of a secretion mechanism.

	I		II	
	Lumen	Blood	Lumen	Blood
Digoxin	24.7± 3.4	61.2± 3.4	56.0± 4.3	31.9± 1.8
Convallatoxol	17.6± 1.6	79.3± 2.9	30.0± 2.0	56.3± 2.3

Tab. Relative uptake of digoxin and convallatoxol. Applied concentration 1nmoles/ml, incubation time 45 min. I oxygen, II oxygen + 2,4-DNP(digoxin) or nitrogen (convallatoxol). Mean ± S.E.M. from 5-21 experiments.

SYMPOSIUM II

Fetal pharmacology

S. Ullberg (Department of Toxicology, University of Uppsala, Biomed. Centre, Uppsala, Sweden). UPTAKE AND DISTRIBUTION OF DRUGS IN THE FETUS.

A number of whole body autoradiograms will illustrate the varying distribution pattern in pregnant animals of drugs such as vitamins, antibiotics and psychopharmacologic agents. The technique used has the advantage that the concentration of a labelled compound can be compared in fetus, placenta and maternal tissues on the same picture and that the detailed distribution within the fetus is revealed. Our material is, however, mainly restricted to late gestation time. In some cases the autoradiographic results are complemented with quantitative measurements and with chemical microseparation studies.

Among the findings may be mentioned that some vitamins and other physiological substances are accumulated in the fetal tissues in relation to the maternal tissues. Vit. B_{12} is exceptional in being concentrated more than 100 times apparently by an active placental transportation mechanism.

The more unphysiologic a substance is the more is its distribution pattern influenced by physicochemical properties such as fat solubility and degree of ionization Fat soluble compounds thus generally pass both to the brain and the fetus and drugs which influence the brain (including thalidomide)rapidly reach maternal levels in the fetuses, while quarternary drugs are blocked by both placental and blood-brain barriers

The distribution pattern within a fetus mostly shows certain similarities with the maternal pattern. However, the fetal picture is generally more even than the maternal. The fetus seems to lack many of the transportation and accumulation mechanisms, which are developed in adult animals There is also, with exception for a few drugs, a lack of accumulation in fetal excretory pathways kidney, urinary bladder, biliary ducts, intestine

A rather frequent finding is a very strong and selective accumulation in one single type of fetal tissue Thus tetracycline accumulates selectively in the fetal skeleton, thiouracil in the fetal thyroid and chloroquine and chlorpromazine in the pigment of the fetal eye.

Some substances seem to cause fetal damage without reaching the fetus. The classical example is trypan blue which is localized in the yolk sac placenta, where it may interfere with a fetal nutritional mechanism An accumulation in the yolk sac placenta is also seen for cortisone and the herbicide 2,4,5-T.

H. J. Merker (II. Anatomisches Institut der Freien Universität Berlin, 1 Berlin 33, Königin-Luise-Str. 15, Germany) THE ANATOMY OF THE FETUS

When discussing the morphology of the embryo or the fetus the following facts have to be taken into consideration 1) The constant and rapid change of the structure of the whole embryo, its parts, and its cells These processes include A. changing localisation of areas with a high mitotic activity, B. differentiation of immature tissues, C. changes in the size of individual parts (liver, brain, lung) in relation to the whole organism, D. alterations of the shape (changing relationship between yolk-sac surface and ectodermal area), and E. change of the mother-embryo barrier (e.g Reichert's membrane-yolk-sac-placenta) - 2) Dependence of the embryo on the mother animal which leads to growth inhibitions of some organs (lung) or growth stimulations (adrenal gland) and forms the circulatory system (umbilical vessels, foramen ovale, ductus Botalli, ductus venosus). - 3) The distinct species-related differences in growth velocity, gestation period, maturation sequence, and formation of the mother-embryo barrier. A great amount of differences in the mode of action of embryotoxic pharmaca can be explained by these differences alone

Under the influence of an embryotoxic pharmacon certain morphogenetic processes are always damaged irreversibly, others only reversibly Here inhibition of induction processes, prevention of motion processes in tissues disturbances in complex and synchronous processes, changes in morphological matrices play an important rôle. As an example for the reasons leading to irreversible lesions palatal closure and the development of the extremities will be discussed.

D Lorke (Institut für Toxikologie der Farbenfabriken BAYER A.G., 56 Wuppertal-Elberfeld, West-Germany):
Methods in Experimental Fetal Pharmacology.

Methods for the evaluation of a possible pharmacological effect of chemical substances on the foetus depend on the stage of gestation. The effect of pharmacological agents can either be observed studying such influence on the recently fertilized ovum or else on the blastocyst. The choice of method depends on the question to be answered.

During a later stage of gestation the influence of a compound on foetal development is usually investigated by treating the maternal animal.

These methods do not differ from those which were developed for the investigation of embryotoxic and teratogenic effects of substances and which are generally recommended.

For the critical evaluation of drug effects the placental passage and foetal distribution are important.

The use of C^{14} labelled substances here are of great help.

A special technique in which the entire uterus is resected at different gestational periods under therapy and cut into slices, can throw more light into the pharmacological effect of drugs on the foetus.

Shortly before delivery drugs which are given to the mother may have an effect on the foetus.

Also for this stage of gestion a special technique allows one to study the pharmacological effect on the foetus.

Anders Rane and Else Ackermann (Department of Pharmacology, Division of Clinical Pharmacology, Karolinska Institute, Stockholm and Department of Clinical Pharmacology, The Medical School, Linköping, Sweden): EVIDENCE FOR DRUG METABOLISM IN THE HUMAN FETAL LIVER. STUDIES IN DIFFERENT CELL FRACTIONS.

Human fetal liver microsomes contains cytochrome P-450 (1) and hydroxylates desmethylimipramine. 60-80 % of the activity of glucose-6-phosphatase and NADPH-cytochrome c reductase remaines in the 200 x g pellet. Electron microscopical studies of this pellet (in collaboration with Dr.J.L. E.Ericsson) revealed the presence of endoplasmic reticulum. Microsomes were observed in the 9000 x g and 105000 x g pellets. The metabolism of aniline (type II substrate) and ethylmorphine (type I substrate) was studied in the 200 x g, 9000 x g and 105000 x g pellets. The greatest oxidation of both occurred in the first two fractions. The metabolism of aniline was of the same order of magnitude as in the microsomal fraction of human adult liver.

Reference

1. Yaffe, S.J. et al. Life Sci. 9, II, 1189-1200, 1970.

H.Uehleke, K.H.Hellmer and O.Reiner (Institute of Pharmacology, University of Tübingen, 74 Tübingen, Wilhelmstr. 56, W.Germany): DIVERGENT PERINATAL DEVELOPMENT OF THE MICROSOMAL N-OXIDATIONS OF INDIVIDUAL TYPES OF AMINES.

In suspensions of liver microsomes of perinatal rats the N-hydroxylation of primary arylamines (4-chloroaniline, p-phenetidine) parallels the development of cytochrome P-450 concentrations and of typical mixed function drug oxidations (C-hydroxylation and dealkylation reactions). In contrast, the microsomal activity for the N-oxidation of N-methylaniline, N,N-dimethylaniline and tertiary aliphatic amines increases rapidly at birth and reaches one peak activity at the age of 2-3 days. Thereupon, the specific activity declines and rises again at the age of about 10 days to reveal a second higher maximum at 27 days. The activities of liver microsomes from rats older than 35 days are greatly influenced by the sex of the animals.
The microsomal activity of glucose-6-phosphatase and of the NADPH cytochrome c reductase develops similar to the N-oxidation of secondary and tertiary amines. The N-oxidations of secondary and tertiary amines have been shown to be not dependent from cytochrome P-450 (H.Uehleke et al.: Z. phys. Chem. 1970, 351, 1475 and 1971, 352, 403; Arch. Pharmak. 1971, 268, 242). The microsomal N-oxidation of several types of arylamines represents a typical toxication reaction. Therefore, the unique development of amine N-oxidation has to be considered in drug therapy during the first life period and in toxicologic and carcinogenic experiments.

D. Neubert, E. Köhler, K. Engels (Pharmakologisches Institut der Freien Universität Berlin, Abt. Embryonal-Pharmakologie", 1 Berlin 33, Thielallee 69/73, Germany) BIOCHEMICAL ASPECTS OF SOME EMBRYOTOXIC AND TERATOGENIC EFFECTS.

Embryotoxic and teratogenic effects can very often be produced with doses which cause only negligible defects within the adult maternal organism. In some respects the situation is similar to that aimed at with an antineoplastic therapy and in fact many cytostatic agents are known to possess the capability of causing embryotoxic or teratogenic effects.

Two reasons may be responsible for the special sensitivity of mammalian embryonic tissue: 1) a higher concentration of the interfering component within embryonic cells than within maternal cells, this being due to either an accumulation or a lack of inactivation or 2) a special susceptibility of embryonic tissue to the toxic agent. - Our group has especially been interested in the second possibility, since we feel that differences in cell metabolism may be responsible for this difference in susceptibility between embryonic tissue and cells of an adult organism. The knowledge of embryonic cell metabolism during the different stages of development and the deviations from metabolic pathways present in adult cells, therefore, would be a prerequisite for the elucidation of the mode of action of embryotoxic and teratogenic agents.

Our group has studied a variety of biochemical parameters and in the first part of this paper some aspects of metabolic pathways in mammalian embryos will be discussed. Special emphasis is laid on growth rate and cell division, nucleic acid metabolism, energy metabolism, and some aspects of protein synthesis.

In the second part of this paper a biochemical analysis of the mode of action of some embryotoxic agents is attempted. Studies on the mode of action of some antimetabolites as well as of alkylating agents and of DNA complexing compounds will be discussed. Furthermore, evidence has been accumulated that thalidomide may possibly exert its teratogenic effect by an interference with collagen synthesis. This is connected with some aspects of ascorbic acid metabolism in mammalian embryonic tissue.

K.S. Larsson[x] (Laboratory of Teratology, Karolinska institutet, S-104 01 Stockholm 60, Sweden): TERATOLOGICAL PROBLEMS

Species and strain differences may cause problems in using experimental animals for screening drugs which must be safe for the human embryo, but, on the contrary, these differences are of value in studies on teratogenic mechanisms. From the view of fetal pharmacology it is of interest that a maternal influence on the frequency of cortisone-induced cleft palate and salicylate-induced fetal death has been demonstrated in two mouse strains. The blastocyst transfer technique has been used in teratological experiments to differ between two types of matroclinous influence; cytoplasmic and uterine environmental effects.

The risk for developmental disturbances during the organogenetic period has been stressed for a long time. Guidelines for a perinatal study have recently been added to existing recommendations for drug tests. The risk for fetal injuries during the late stage of pregnancy has been shown in our laboratory for several years using salicylates as model substances in two mouse strains. In addition to fetal death and hemorrhages, biochemical impairment was demonstrated by changes in fetal prothrombin time and tissue glycogen concentration. It was possible to modify the fetal damaging effect of salicylates by some genetic and environmental factors. There is a need for future cooperation between teratologists and pharmacologists in this particular field. In addition to these specific research problems a teratologist's views on guidelines for testing drugs and other chemicals and problems of documentation will be presented.

F R E E   C O M M U N I C A T I O N S

A P P E N D I X

specially indexed on page 110.

N.-E. Andén[x)], J. Engel, and A. Rubenson (Department of Pharmacology, University of Göteborg, Sweden). SOME ASPECTS OF THE CENTRAL ACTION OF L-3,4-DIHYDROXYPHENYLALANINE (L-DOPA).

The dopamine (DA) and noradrenaline (NA) formed from L-DOPA in the caudal part of the transected spinal cord produce an increase in the hindlimb flexor reflex. In the acutely spinalized rat, the effect seemed to be due to release of endogenous NA since it was not observed after depletion of the NA stores by reserpine and it was accompanied by a reduction of the NA concentration. After inhibition of the monoamine oxidase by nialamide, L-DOPA evoked an increase in the flexor reflex activity also after reserpine treatment concomitantly with accumulation of both NA and DA. Both the NA nerve terminals and the capillary walls contain L-DOPA decarboxylase activities, which were selectively reduced by chronic spinalization and by α-methyldopa hydrazine, respectively. In chronically transected cords, L-DOPA induced DA formation and increased flexor reflex activity in the presence but not in the absence of capillary wall decarboxylase activity. After pretreatment with reserpine, nialamide and α-methyldopa hydrazine, there was a flexor reflex increase only in the acutely operated rats, mainly due to accumulation of NA. Thus, both the capillary and the neuronal decarboxylase activities can be of functional importance for the L-DOPA actions.

S. Aro and E. Klinge[x)] (Department of Pharmacology, University of Helsinki, Finland): EFFECT OF SOME BETA-ADRENERGIC BLOCKING COMPOUNDS ON CARDIAC CHOLINESTERASE ACTIVITY

Cholinergic mechanisms can reduce conduction velocity in various types of cardiac tissue and beta-adrenergic blockers can abolish certain arrhythmias independently of their beta-receptor blocking activity (Fitzgerald, J.D. · Clin. Pharmacol. Ther 1969, 10, 292-306). In the present study the potency of dl-dichloroisoprenaline, dl-pronethalol, dl-propranolol, dl-alprenolol and dl-oxprenolol to block cholinesterase activities of whole heart homogenates of four rats was compared to that of quinidine, procainamide and succinylcholine The manometric technique was employed and the substrates acetylcholine, butyrylcholine or acetyl-β-methylcholine were used in 6×10^{-2} M final concentration. A 10^{-3} M concentration of quinidine inhibited the hydrolysis of acetylcholine by 71%. The same degree of inhibition was produced by a 10^{-5} M concentration of physostigmine. If the potency of quinidine is indicated by 100, the relative potencies of the other compounds were: oxprenolol 82, alprenolol 52, propranolol 35, dichloroisoprenaline 31, pronethalol 21, procainamide 16 and succinylcholine 11 The hydrolyses of butyrylcholine and acetyl-β-metylcholine were inhibited in approximately the same ratio, oxprenolol being in both cases the most potent inhibitor of the beta-adrenergic blockers studied It seems that among these compounds the anticholinesterase activity of the phenoxypropanolamine derivatives is somewhat higher than that of the phenylethanolamine derivatives. It remains to be solved if any of the known clinical properties of the beta-adrenergic blocking compounds can be attributed to their slight anticholinesterase activity.

Grant: The Yrjö Jahnsson Foundation, Helsinki

H Coper, M Fernandes, H Honecker, S Kluwe (Institut für Neuro-psychopharmakologie der Freien Universität, 1 Berlin 19, Ulmenallee 30 Germany) THE INFLUENCE OF SOLVENT AGENTS ON THE EFFECTS OF CANNABIS

One dose of cannabis resin (containing 10 mg/kg THC 9 mg/kg Cannabinol , 20 mg/kg Cannabidiol , about 1 mg/kg unknown substances) alters hexobarbital sleeping time, body temperature and catalepsy of male rats The influence of different solvent and suspension agents of cannabis on these parameters were recorded The intensity and duration of action of cannabis suspended either in polyethyleneglycol (60 Vol. % in 0 9 % NaCl) or propyleneglycol (30 Vol % in gummi arabicum) was not significantly different A single injection of THC did not enhance hexobarbital sleeping time after 24 hours The body temperature (recorded at 22°C room temperature) was decreased from 38°C to 35°C one hour p i and returned to normal within 24 hours Catalepsy could also be observed for up to 6 hours However, cannabis resin when given in rape oil did not produce hypothermia or catalepsy Hexobarbital sleeping time on the other hand was found to be increased even after 4 days of a single injection of THC (from 39 min to 63 min $p < 0,01$) Our experimental data suggest that the pharmacokinetic of cannabis suspended in various agents might be responsible for the observed variability in all parameters studied

M Fernandes D Rating S Kluwe (Institut für Neuropsychopharmakologie der Freien Universität, 1 Berlin 19 Ulmenallee 30 Germany)
THE INFLUENCE OF SUBCHRONIC TETRAHYDROCANNABINOLE- AND CANNABISTREATMENT ON FOOD- AND WATER-INTAKE BODY WEIGHT AND BODY TEMPERATURE OF RATS

A single i p injection of an extract of cannabis resin (containing 5 mg/kg THC, 4,5 mg/kg Cannabinol , 10,5 mg/kg Cannabidiol , and 0 5 mg/kg unknown substances) reduced the body weight of male rats The daily intake of food and water was also reduced by 20 and 25 % respectively Daily injections of cannabis (10 to 12 a m) causes a maximum inhibition of food and water intake by the 4th to 6th day and returns to its normal level on the 11th day Similar observations were recorded after treatment of rats with 5 mg/kg 8-THC The loss of body weight and the reduction c' [illegible] reached its maximum on the 2nd day [illegible] ‹l level on the 8th day The effect of [illegible] The cannabis resin at the [illegible] of orally [illegible] after ten [illegible] temperature of rats [illegible] marked when [illegible] 36 4°C on

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level on the 11th day Similar observations were recorded after treatment of rats with 5 mg/kg ^{8}-THC The loss of body weight and the reduction of food intake caused by THC reached its maximum on the 2nd day of treatment and returned to control level on the 8th day The effect of THC on water-intake has not been recorded. The cannabis resin at the same dose level in this experiment delayed the excretion of orally administered tap water However, this effect was abolished after ten daily injections

Intra peritonial injection of Δ^{8}-THC lowered the body temperature of rats from 37 8°C to 35 5°C at 90' p. i This effect was less marked when the rats were treated subchronically with THC (36.0°C and 36.4°C on the 5th and 10th day respectively, $p < 0$ 001)

K.Hashimoto[x)], N.Taira, T Iijima and K Hashimoto,Jr. (Department of Pharmacology, Tohoku University School of Medicine, Sendai, Japan) EFFECTS OF BETA-ADRENERGIC ANTAGONISTS ON AV CONDUCTION.

Effects of beta-adrenergic antagonists on AV conduction were investigated in vitro on canine AV node preparations cross-circulated with arterial blood from donor dogs through the cannulated right coronary, AV node and anterior septal arteries. The preparation consisted of the right atrium and the ventricular septum, and thus included the main conducting system. With pacing the right atrium at 2.5 Hz, intervals between atrial and ventricular bipolar electrograms were continuously registered by means of an AV interval-graph. Close-arterial injection of 0.1 to 0.3 nmol of l-noradrenaline (l-NA) into the AV node artery produced an evident positive dromotropic effect. This positive dromotropic effect was antagonized by dl-pindolol, l-propranolol and l-alprenolol in doses 10 times those of l-NA, and by dl-sotalol in doses 100 times those of l-NA. In contrast, d-propranolol exerted no significant antagonistic effect even in doses 1000 times those of l-NA. In doses of 30 to 300 nmol all of these antagonists per se exerted a biphasic negative dromotropic effect, consisting of a transient and a long-lasting and progressive one.

H.Marquardt*, E.Huberman, P.Grover, T.Kuroki, P.Sims, and Ch.Heidelberger (McArdle Laboratory for Cancer Research, Madison, Wisconsin, U.S.A.)

K-REGION DERIVATIVES OF HYDROCARBONS. MALIGNANT TRANSFORMATION AND STIMULATION OF DNA SYNTHESIS IN VITRO.

The activity to induce malignant transformation in vitro of K-region derivatives (epoxide, dihydrodiol, phenol) of the polycyclic aromatic hydrocarbons 1,2-benzanthracene (BA), 1,2,5,6-dibenzanthracene (DBA) and 3-methylcholanthrene (MCA) was tested. A particular clone of mouse prostate cells (Chen and Heidelberger, Int. J. Cancer 4, 166, 1969) which did not metabolize hydrocarbons to a great extent was not transformed by BA or MCA, their respective epoxides induced a high yield of transformation. N-methyl-N'-nitro-nitrosoguanidine which is active without prior metabolism also induced transformation In hamster embryo cells (Berwald and Sachs, J. Natl. Cancer Inst 35, 641, 1965) BA, DBA, and MCA did induce transformation, their respective epoxides, however, were much more active. In hamster embryo cells dihydrodiols were nearly as active as epoxides. The data support the suggestion that hydrocarbons need metabolism prior to their carcinogenic activity

In order to elucidate the mechanisms involved in chemical transformation in vitro the effect of K-region derivatives of BA on DNA synthesis in growing hamster embryo cells was investigated The two very active transforming derivatives, epoxide and dihydrodiol, stimulated DNA synthesis (between 3-24 hrs after application) This has also been demonstrated in cells after infection with DNA containing tumor virus Stimulation of cell DNA synthesis may be an event required for the fixation of the transformation process.

A Pekkarinen University
Turku,Finland BY COMBINED
TREATMENT WITH 11-343 A)
AND 2 % NaCl BY DIURET-
IC,α-SYMPATHO-

Treatment with DMPP (20 mg/kg p.o.daily) and 2% NaCl in drinking water increased the mean blood pressure (=BP) in 4 groups of 10 rats to the maximum (148.5+1.7-157.5+1.7 mmHg) in 95-109 days with +36.0,+32.0,+37.5 and +42.0 mmHg respectively ($p<0.001$), and was then continuously elevated with a tendency to decrease later in 5-6 months from the beginning. A smaller dose (10 mg/kg) increased BP to 144.4+1.8 mmHg (30.0 mmHg) in 70 days ($p<0.001$).McNeil-343 A (10 mg/kg p.o.daily) increased BP to 149.5+1.2 and 148.5+2.0 mmHg in 140-129 days
(+35.0 and +36.0 mmH
celerated the increa
(+43.8 mmHg)($p<0.001$
ed the increase of B
($p<0.001$).Furosemide
(10 mg/kg)(+11.0)as
and thymoxamine (12
hypertensives: reser
kg)(+9.0),guanoxan (
kg)(+10.7), α-methyldo
kg)(+8.0),clonidine
with reserpine (3 mg
(+11.5),as well as c
hibited the BP increase during DMPP (20 mg/kg) and 2% NaCl
treatment ($p<0.001$). α-MPT and FLA-63 decreased the BP of DMPP

D. Rating, H. Honecker, I Broermann, S Strauss (Institut für Neuropsychopharmakologie der Freien Universität, 1 Berlin 19, Ulmenallee 30 Germany): HEXOBARBITAL-SLEEPING TIME AND AMPHETAMINE MOTILITY AFTER SUBCHRONIC TETRAHYDROCANNABINOL-TREATMENT

5 mg/kg Δ^8-THC i. p. prolongs hexobarbital-sleeping time to 205 min (control = 119 min, p 0, 0027). The enhancement of hexobarbital-sleeping time by THC is significantly reduced after 9 days treatment with 5 mg/kg THC (156 min, p 0, 0027). Acute or subchronic treatment of THC did not alter the elimination of hexobarbital from brain or blood of rats. d-amphetamine-sulphate (0. 2-2. 0 mg/kg) enhanced locomotory and exploratory activity in rats, while higher doses (5, 0-10. 0 mg/kg) lead to (60-150 min p. i.) a period of strong stereotyped behaviour without any locomotion; the rats are "fixed" The stereotyped behaviour is preceded and followed by the above mentioned enhanced locomotory and exploratory activity. Using a photocellactivity-box the "fixation" was found to be dose related 10 mg/kg ^{8}-THC shifts the dose-response-relationship towards lower amphetamine doses. [THC-ED_{50} = 2. 40 mg/kg (2. 05-2. 88), Control ED_{50} = 3. 44 mg/kg (3. 08-3. 79)] . This effect may be interpreted as an enhancement of amphetamine effect. However, after ten-days-treatment with 10 mg/kg Δ^8-THC this enhancing effect is almost abolished. Neither enhancement nor abolition of THC effect can be explained by an altered amphetamine elimination.

D.Brdberg,[x] M.Kelly, A.-L. Myrsten, A. Neri and S.-G. Servais
(Dept. of Alcohol Research, Karolinska Institutet, S-104 01 Stockholm, Sweden: ACUTE EFFECTS AND AFTER-EFFECTS OF ALCOHOL ON PHYSIOLOGICAL AND PSYCHOLOGICAL FUNCTIONS.

Groups of non-alcoholic subjects participated in experimental sessions lasting for until 20 hr. Alcohol was in in one session administered in doses of 0.72 or 1.43 g/kg. In the second session the subjects served as their own controls and drank only mineral water. Changes in objective and subjective variables were ascertained at fixed intervals. During the intoxication phase significant changes were found in pulse rate, standing steadiness, hand steadiness, chioce reaction time, Spoke test A and B, in the verbal, numerical, inductive and spatial factor test, and in the correction test. At the same time the subjects displayed positional alcohol nystagmus phase I and rated themselves as intoxicated, elated, talkative and less sleepy than "normal". Moreover, the diuresis and the urinary excretion of catecholamines and electrolytes were influenced. In the post-alcohol phase significant deviations from control were recorded in pulse rate, systolic and diastolic blood pressure, standing steadiness, hand steadiness, spatial factor test and in the correction test. During the period an alcohol nystagmus (PAN II) was recorded, as well as an impairment of diuresis, urinary excretion of adrenaline and electrolytes, and the subjects stated they felt drowsy, tired, sleepy, dizzy and thirsty. 93

Ref.: Kelly et al.: Blutalkohol, 1970, 7, 422-36. Myrsten and Rydberg: Alkoholfrågan, 1970, 1, 11-18. Myrsten et al: Rep. Psychol. Lab. Univer. Stockholm, 1970, 314, 23 pp.

A.Windorfer*,W.Lehnert and W.Künzer(University Children's Hospital,Freiburg i.Brg.W-Germany):ENTERAL RESORPTION OF C-14-HAEMOGLOBINE.

The resorption of porphyrine from the duodenum was studied in rats using a solution of C-14-tagged haemoglobin.The tagging was carried out by incubation of duck erythrocytes with C-14-Delta-Aminolaevulic-acid,resulting in an incorporation quotient of 12%.A comparative test with C-14-glycine resulted in a 27o%higher incorporation quotient.The tagged haemoglobin was given by duodenal gavage and the bile collected by a bile-duct fistula.The resorption rate for haemoglobin or porphyrine was calculated from the total radioactivity found in the bile.It was 2,5-3,2% of the applied dose.

AUTHOR AND SUBJECT INDEX OF FREE COMMUNICATIONS

Drug Code Numbers

See also index to appendix on page 110.

ACTA PHARMACOLOGICA ET TOXICOLOGICA

VOLUMEN 30, SUPPLEMENTUM 1. 1971

ANAPHYLACTIC HISTAMINE RELEASE AND *INFLUENCE OF ANTIRHEUMATICS*

BY

SVEND NORN

MUNKSGAARD COPENHAGEN 1971

Anaphylactic Histamine Release
and
Influence of Antirheumatics

Forsvaret finder sted onsdag den 6. oktober 1971 kl. 14 præcis
i Danmarks Farmaceutiske Højskoles auditorium 4

ACTA PHARMACOLOGICA ET TOXICOLOGICA

Volumen 30, Supplementum I

Anaphylactic Histamine Release and Influence of Antirheumatics

By

Svend Norn



MUNKSGAARD COPENHAGEN 1971

Printed in Denmark by P. J. Schmidt, Vojens

To my wife

Denne afhandling er sammen med de syv tidligere publicerede afhandlinger af Danmarks farmaceutiske Højskole antaget til offentligt at forsvares for den farmaceutiske doktorgrad

København, den 7 juli 1971

Helmer Kofod
rektor

Preface

The initial steps of the experimental studies which form the basis of this publication were taken in the Pharmacology Department of H. Lundbeck & Co Ltd., Pharmaceutical Manufacturers, Copenhagen During this stage I received *great encouragement* through the positive attitude of J MØLLER NIELSEN, D V.S The work was continued in the Institute of Pharmacology, University of Copenhagen, whose former head, Professor KNUD O MØLLER has afforded unfailing help and interest. I also wish to express my deep gratitude to Professor JENS S SCHOU, the present head, for his stimulating support and invaluable advice

Quite particularly, I extend my thanks to KLAUS JENSEN, M D, Blegdamshospitalet, Copenhagen, who rendered invaluable support by his unique advice in critical phases. For greatly inspiring discussions my thanks go to Professor TORKELL JÓHANNESSON, Reykjavik, and to Professor ERIK JACOBSEN, Copenhagen

For perusal of the manuscript I am indebted to HANS LANGGÅRD, M D, Copenhagen, and for practical advice to my colleagues, especially cand scient. J AAS JANSEN, and cand. pharm. H. H. LAUSEN

The energetic work of Mrs. JUDITH RASMUSSEN, H Lundbeck & Co, Mrs. ANNELISE HONIG, and Miss ANNETTE FRANCKER OLSEN, University Institute of Pharmacology, is gratefully acknowledged.

To the Danish Science Foundation I am indebted for having received the expenses of technical assistance through three years, and to Statens Seruminstitut, Copenhagen, for having supplied me with vaccine.

Copenhagen, August 18, 1969

SVEND NORN

Previous Publications

A. Norn, S Chemical determination of histamine in tissues and mast cells
Acta pharmacol et toxicol 1965b, 22, 115–125

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Contents

Chapter 1

Background

Histamine was synthesized in 1907 by WINDAUS & VOGT, and was demonstrated in animal tissue by BARGER & DALE in 1911 A relationship between histamine and anaphylaxis was suspected at an early date (DALE & LAIDLAW 1910 DALE 1913) because of the similarity between the pharmacological actions of histamine and the symptoms of anaphylactic reaction But it was not proved until 1932 that histamine – or a histaminelike substance – is released from the tissues during anaphylaxis Thus, in the dog an increased histamine content was demonstrated in the blood (DRAGSTEDT & GEBAUER FUELLNEGG 1932, GEBAUER FUELLNEGG & DRAGSTEDT 1932) and a reduced content in the liver (AKCASU & WEST 1960) in anaphylactic shock. A similar increase in the histamine content of the blood was found in the rat (CODE *et al* 1961, MOTA 1957) and in the guinea pig (SCHMUTZLER *et al* 1963)

Later on it was shown that the tissue content of histamine was located in the mast cell In the first place, RILEY & WEST (1953) demonstrated that a positive correlation exists between the histamine content and the mast cell population of various normal and abnormal tissues in various animal species Furthermore, PARRATT & WEST (1957) demonstrated that repeated administration of the histamine liberator polymyxin B depletes rat skin almost entirely of mast cells, and at the same time the cutaneous tissue becomes practically devoid of histamine Gradually as the mast cell population regenerated, the histamine content of the skin also re attained its normal level In other words, by far the greater part of the histamine in the body is deposited in the mast cells and very little histamine is free Minor quantities are bound in blood cells and platelets (VALENTINE *et al* 1955 GRAHAM *et al* 1955, HUMPHREY & JAQUES 1954), but these histamine depots probably play no quantitative role in anaphylaxis That histamine is in fact released from the mast cells during anaphylaxis has been demonstrated indirectly by degranulation of the cell in intact animals (MOTA 1958 AKCASU & WEST 1960)

The released histamine is a factor in eliciting the anaphylactic reaction, since anaphylactic shock is counteracted if the animal has been treated with antihistamines or histamine liberators prior to the antigen challenge (GREEN 1953, MOTA 1967, AKCASU & WEST 1960) However, the role

of histamine appears to differ from species to species, being for instance more pronounced in the guinea-pig than in the rat Thus, anti-histamines can protect the guinea-pig from fatal shock, while the rat can be protected only from nonfatal shock (GREEN 1953, MOTA 1957 & 1963, SANYAL & WEST 1958) In addition, the rat is far less sensitive to *administration of* histamine than is the guinea-pig (NARANJO 1966) Accordingly, in the rat other factors must be contributory to the fatal anaphylactic shock These factors might be serotonin (5-hydroxytryptamine), SRS-A (slow-reacting substance in anaphylaxis) and kinins, all of which are released during the anaphylactic reaction (UNDENFRIEND & WAALKES 1959, SANYAL & WEST 1958, BROCKLEHURST 1960, CHAKRAVARTY & UVNAS 1960, COLLIER & JAMES 1967, BROCKLEHURST & LAHIRI 1962 & 1963) Another possible factor might be cellular hypersensitivity reactions (delayed-type) (p 18)

Various antirheumatic substances are known to protect animals from fatal anaphylactic shock Thus, when administered prior to challenge with antigen, cortisone protects the rat and the mouse (CODY *et al* 1963, MALKIEL & HARGIS 1952, NELSON *et al* 1950), while it does not protect the guinea-pig (DWORETZKY *et al* 1950, ARBESMAN *et al* 1951) Similar treatment with acetylsalicylic acid, sodium salicylate, and amidopyrine affords protection in the rabbit (LEPPER *et al* 1950, CAMPBELL 1948), while salicylate does not protect the guinea-pig (SMITH & HUMPHREY 1949) Conflicting results have been obtained with phenylbutazone administered daily to the guinea-pig throughout the sensitization period Thus, BERTOLA & CASTELLANI (1958) could protect all the animals from fatal shock, while TIGANO (1955) found no change at all in the incidence of fatal shock

The role of histamine in anaphylactic shock and the ability of antirheumatics to counteract this shock motivates a study of whether these drugs will influence the release of histamine from the mast cells during anaphylaxis The rat was selected as the experimental object, because glucocorticoids protect this species from fatal anaphylactic reaction An accurate measure of the histamine release from the mast cells cannot be obtained by *in vivo* experiments because of the distribution of histamine in the organism, its metabolization, and excretion Therefore, the release of histamine was studied by *in vitro* experiments, incubating peritoneal mast cell suspension from sensitized rats with specific antigen

The object of the present study can thus be formulated in the following items

(1) The author has primarily aimed at *working out an* in vitro *method* for the quantitative determination of histamine release in anaphylactic reaction This method should be sufficiently specific and sensitive, and the histamine release should be effected by a specific antigen antibody reaction

(2) Secondly, it was endeavoured to *elucidate the mechanism of the ana-*

phylactic histamine release, int al and particularly, by investigating the effect which pre treatment with various antirheumatic agents would have on this mechanism

The present studies were based exclusively on experimental investigations on the rat However, there seem to be certain differences in the individual steps of histamine release in various animal species These differences *will be discussed in relation to investigations on the guinea pig* made by other authors, and the consequences on the general validity of the results obtained will be discussed in chapter 5

Chapter 2

Anaphylactic Histamine Release

Introduction

In active sensitization the animal is exposed to the action of a foreign protein (antigen) In response, the organism forms an *antibody* specific to the antigen concerned If the animal is again exposed to the same antigen at a time when sufficient quantities of antibody have been formed, the antigen will react with the antibody in a specific *antigen-antibody reaction* As concerns the mast cell-sensitizing antibody (p 18), the antigen-antibody reaction is connected to the mast cell, since the antigen reacts with the antibody which is bound in the mast cell, or to its cell surface (MOTA & DIAS DA SILVA 1960, HUMPHREY & MOTA 1959) Therefore, the reaction can also be elicited *in vitro* by adding antigen to mast cells from sensitized animals The reaction does not require complement in the rat and in the guinea-pig (GREAVES & MONGAR 1968, BECKER & AUSTEN 1964 & 1966) The antigen-antibody reaction elicits degranulation of the mast cell In the granula of the mast cell, the histamine content of the cell is electrostatically bound to protein-heparin complexes (ÅBORG *et al* 1967), and the histamine is formed in the mast cell by decarboxylation of histidine (TELFORD & WEST 1963) By the mast cell degranulation the granules are released and can be demonstrated scattered outside the cell in the rat, but not in the guinea-pig (MOTA 1963b) Presumably this takes place in the rat without major destruction of the cell membrane and without any major change in the metabolic function of the cell – at low concentrations of antigen Thus, slight swelling of the cell and re-distribution of its granules have been observed, and judging by the respiratory rate, the metabolic activity of the cell remains unchanged except for a brief period with an initial increase (MOTA 1963b, CHAKRAVARTY 1968) The *biochemical mechanism* of the mast cell degranulation is unknown, but several theories have been advanced (*cf* DIAMANT 1962) It should be emphasized, in particular, that enzymatic and energy-yielding processes seem to be prerequisites of degranulation and histamine release This has been demonstrated indirectly by the influence exerted upon the release of granules and histamine by pH, temperature, ionic environment (Ca^{++} deficiency) enzyme inhibitors in oxidative metabolism, anoxia, and glucose in anoxia (*cf* 24, 25) Recently DIAMANT found that ATP liberated histamine from the mast cell, and that an "ecto-ATP-ase" was located in the mast cell membrane

(DIAMANT & KRUGER 1967, DIAMANT 1969) These findings might lead to a more profound understanding of the biochemical mechanism With the granules outside the mast cell, histamine is passively released from the granule-complex during an exchange with cations (UVNAS & THON 1966) The released histamine is eliminated mainly by oxidative deamination and methylation (TELFORD & WEST 1963)

From the above, it appears that the anaphylactic histamine release includes the following main phases (a) antibody production, i e formation of mast cell sensitizing antibody, (b) antigen antibody reaction, and (c) biochemical mechanism

Active sensitization

The ability to develop anaphylactic hypersensitivity differs from species to species The guinea pig is easily sensitized to various antigens without the use of adjuvant, challenge with specific antigen eliciting fatal anaphylactic shock (DWORETZKY *et al* 1950, GREEN 1953) During the anaphylactic reaction release of histamine can be demonstrated *in vivo* (SCHMUTZLER *et al* 1963) as well as *in vitro* (CHAKRAVARTY 1960, MONGAR & SCHILD 1958) Unlike the guinea pig, the rat is difficult to sensitize Even repeated injections of an antigen will give rise to only transient symptoms of shock at later challenge with antigen (HALPERN *et al* 1955) The same applies to prolonged exposure to alum precipitated antigen (MOTA 1957) In contrast to this a stronger sensitization is obtained when administration of the antigen is supplemented, at the same time, by Freund s adjuvant i e killed *Mycobacterium tuberculosis* suspended in a water in-oil emulsion After sensitization challenge with antigen elicits mild to severe symptoms of shock, and about half the rats die (LIPTON *et al* 1956) However, the strongest and most reliable sensitization is obtained by administering *Bordetella pertussis* bacteria (*Haemophilus pertussis*) as an adjuvant at the same time as the antigen A single dose will sensitize better than several doses Challenge of the sensitized rats will elicit fatal shock in practically all animals (MALKIEL & HARGIS 1952) During this shock the plasma level of histamine rises and microscopic examination of various tissues shows that the mast cells have released their granules (MOTA 1958) Peritoneal mast cells from the sensitized animals show distinct release of histamine on incubation with specific antigen *in vitro* (NORN 1967a & 1968a) This method of sensitization therefore appears well suited for investigating anaphylactic release of histamine It was employed in the present author s studies

Chapter 2

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release varied a good deal from experiment to experiment within the control group (table 7), but the variations within the individual experiment were sufficiently small for studying the effect of drugs upon the anaphylactic release of histamine

The guinea pig differs in several respects from the rat. In the first place it easily develops anaphylactic hypersensitivity to various antigens without the use of adjuvant (p 17) Furthermore, the antibody found in the serum of these animals which is responsible for passive systemic anaphylaxis as well as passive sensitization of isolated tissue in the guinea pig (BENACERRAF et al 1963, OVARY et al 1963, MOTA 1963b, STECHSCHULTE et al 1967, BENACERRAF 1968) is not identical with the mast cell sensitizing antibody in the rat, differing from it in being *int al* precipitating and heat stable at 56° (BENACERRAF 1968)

Methods

For investigating anaphylactic histamine release *in vitro*, any tissue from the sensitized animal is in principle applicable, since practically all tissues contain mast cells The tissue is cut into small pieces, suspended in a Tyrode solution, and incubated at 37° with specific antigen After centrifugation, the quantity of released histamine is determined in the cell free supernatant and expressed as per cent of the total histamine content of the sample Nearly all tissues from the guinea pig show a similar percentage of histamine release, but a few, such as spleen liver, and intestine, show a marked divergence (CHAKRAVARTY 1960) The tissues which have most often been used for studying anaphylactic histamine release are mesenterial tissue and pulmonary tissue Investigations of tissues do not afford a possibility of studying the mast cell isolated Therefore, the influence of other cells and substances upon the histamine release cannot be considered separately Another disadvantage is that the injury to the tissue in cutting it up into small pieces may cause a fairly marked spontaneous histamine release This disadvantage is reduced when using isolated, but intact tissues perfused with Tyrode's solution The antigen is introduced by injection into the perfusion solution flowing to the tissue By this technique, BROCKLEHURST (1960) has demonstrated anaphylactic histamine release in pulmonary perfusate from various animal species The explanation why this could not be demonstrated in the rat is that in this species the sensitization was less effective owing to the use of alum precipitated antigen (*cf* p 17) The drawback of this technique is partly that it gives occasion for only one experiment on each animal, and partly that it is cumbersome and time-consuming. To obtain a simpler method for investigating anaphylactic histamine release, several authors have used suspensions of peritoneal cells obtained by irrigating the rat peritoneal cavity

Mast cell-sensitizing antibody

Serum from sensitized rats has shown two types of antibody of particular interest in anaphylaxis One is *precipitating and will be referred to as* precipitating antibody The other antibody is characterized by being able to cause passive sensitization of isolated rat tissue so that subsequent exposure to antigen releases histamine from the mast cells (MOTA 1963c) This antibody is, therefore, called mast cell-sensitizing antibody, mast cell-lytic, or anaphylactic antibody Rats, sensitized with antigen plus Freund's adjuvant, show a high serum level of precipitating antibody, but a low level of mast cell-sensitizing antibody Exactly the reverse applies to rats sensitized once or a few times with antigen plus *Bordetella pertussis* as an adjuvant After this sensitization, provocation with antigen leads to severe anaphylactic shock, nearly always with a fatal outcome, and to marked degranulation and release of histamine from the mast cells (MALKILL & HARGIS 1952, MOTA 1958 & 1964 & 1968) These reactions are much weaker in rats sensitized with antigen plus Freund's adjuvant Thus, the shock is rarely fatal, and the release of histamine is slight and less common (LIPTON *et al* 1956, MOTA 1961 & 1964 & 1968) Passive systemic anaphylaxis *in the rat shows similar appearances, i e precipitating antibodies* give rise to only a mild shock without release of histamine, whereas mast cell-sensitizing antibody can elicit fatal shock with generalized mast cell degranulation (MOTA 1962 & 1964b) Accordingly, it is likely that mast cell-sensitizing antibody is responsible for the histamine release as well as for the anaphylactic shock in the rat However, other factors must contribute to the fatal shock MOTA (1964b) found several indications of cellular hypersensitivity as another contributory factor Thus, passive sensitization by intravenous administration of rat serum containing mast cell-sensitizing antibody will lead to fatal shock at later challenge with antigen only if cellular hypersensitivity is present at the same time in the recipient rat In the absence of cellular hypersensitivity the shock will be only mild and transient, even though there is generalized mast cell degranulation In the same way, the named fatal shock in rats, actively sensitized with antigen plus *Bordetella pertussis*, cannot be elicited until at a time when the animals exhibit cellular hypersensitivity MOTA, therefore, assumes that fully developed anaphylactic hypersensitivity is present in the rat only when both mast cell-sensitizing antibody and cellular hypersensitivity are present

In respect to the practical performance of passive sensitization by mast cell-sensitizing antibody, it should be mentioned that by intraperitoneal administration, NORN (1968b) obtained heavy sensitization of the peritoneal mast cells of the recipient rat by merely 2 ml donor serum The sensitization was measured by anaphylactic liberation of histamine (p 35) The histamine

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the cell suspension revealed that the spectrophotometric activity is localized exclusively in a zone with an R_F identical to that of histamine Furthermore, the histamine content per mast cell found by NORN s modified method is in complete agreement with the results obtained by other methods, such as biological assay (guinea pig ileum) and spectrofluorometric determination (SHORE *et al* 1959) as is apparent from table 1 In this connection it may be mentioned *that the histamine* content of the peritoneal cell suspension originates almost exclusively from the mast cells as mentioned below

SHORE *et al* (1959) have worked out a sensitive method in which inter- [illegible] substances are eliminated by extraction of the histamine which is then [illegible] ne- [illegible] in [illegible] various tissues but subsequent studies have shown that the brain contains [illegible] substances (CARLINI & GREEN 1963 KREMZNER & PFEIFFER [illegible] the present study is based NORN (unpublished results) could measure histamine quantities as small as 0 10 μg histamine base per 3 50 ml sample which is sufficient sensitivity, as the peritoneal cell suspension contains between 0 5 and 3 μg histamine base per ml (NORN 1968a) The fluorescence intensity was found proportional to a histamine concentration over the range 0 03 to 5 μg per ml and recoveries of histamine added to the cell suspension range from 95 to 100 % (NORN, unpublished results) The method must be considered specific for the assay of histamine in rat peritoneal cell suspensions as the histamine content per mast cell has proved to be in complete agreement with results obtained by means of other methods (table 1)

Histamine content of the peritoneal mast cell

A suspension of peritoneal cells from actively sensitized rats contains, among a number of different cells, about 3 % mast cells The other cells are chiefly lymphocytes eosinophilic leukocytes and mesothelial cells (NORN 1968a) The histamine content of the cell suspension however, is derived almost exclusively from the mast cells This has been proved by a high positive correlation between the concentration of histamine and the concentration of mast cells The correlation was found in peritoneal cell suspensions as well as in fractionated samples thereof containing only a few leukocytes (NORN 1968a) The content of histamine in peritoneal mast cells was found by NORN (1965a & 1968a) to be as a rule between 20 and 40 μg histamine base per 10 mast cells in sensitized as well as in non sensitized rats This is in conformity with previous findings in isolated and non isolated peritoneal mast cells from non sensitized rats (table 1)

with Tyrode's solution (UVNÄS & THON 1959; BRAY & ARSDEL 1961; PERERA & MONGAR 1963 & 1963b; NORN 1965a & 1967b). This method was used in the present study. Here only a few of the immediate advantages of the method will be mentioned, *viz.* that the mast cell-concentration can be determined by counting in a counting chamber after staining with toluidine blue (LAGUNOFF & BENDITT 1959; BRAY & ARSDEL 1961), and that little spontaneous histamine release occurs (p. 24). Below (pp. 20–24, 26) will be mentioned investigations showing that this method fulfills requirements which should be made on a method for assessing anaphylactic histamine release.

Histamine assay.

Quantitative determination of histamine in blood and tissues was previously made chiefly by biological assay based upon contraction of the guinea-pig ileum and the fowl's rectal caecum, as well as upon the decrease in the cat's blood pressure (BARSOUM & GADDUM 1935, CODE 1937; FELDBERG & KELLAWAY 1937, FELDBERG & TALESNIK 1953). These methods are sufficiently sensitive, and with the use of various antagonists, such as atropine, mepyramine, and the serotonin antagonist BOL 148 (2 bromo-d-lysergic acid diethylamide) a fairly high specificity is obtained. However, a content of interfering substances has been found in the brain, and this necessitates preliminary purification of the tissue extract prior to the assay (CARLINI & GREEN 1963).

Chemical methods have in certain cases simplified the determination of histamine. Two of these methods will be mentioned, because they show relatively high specificity and sensitivity. LOWRY *et al.* (1954) coupled 2 4-dinitrofluorobenzene to histamine, and the yellow coupling product was assayed spectrophotometrically after removal of interfering substances by passing the sample through a Decalso column and extracting with an organic solvent. However, certain amines still interfere with the analysis In this connection there is special reason to mention the polyamine spermidine, because it has been found in various body tissues (TABOR & TABOR 1964) The method has been adapted for determination of histamine in semi-micro scale instead of in micro scale by NORN (1965b) for assay in rat peritoneal cell suspensions in the initial experiments (NORN 1965a) included in the present study. The modified method is sufficiently sensitive for measuring histamine in peritoneal cell suspensions, and there are several indications that determination in this cell suspension is specific, unlike that in certain other tissues (NORN 1965b). For instance, treatment of the peritoneal cell suspension with histaminase (diamine oxidase, *cf.* TABOR & TABOR 1964) completely destroyed the spectrophotometric activity, and this excludes a content of measurable quantities of interfering polyamines. Paper chromatograms of

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SHORE *et al* (1959) have worked out a sensitive method in which interfering substances are eliminated by extraction of the histamine which is then coupled to o-phthalaldehyde and assayed spectrofluorometrically This method has previously been considered extremely specific for measurement in various tissues but subsequent studies have shown that the brain contains interfering substances (CARLINI & GREEN 1963 KREMZNER & PFEIFFER 1966) With negligible modifications (NORN 1967b) the method was used by NORN for assay in peritoneal cell suspensions in experiments upon which the present study is based NORN (unpublished results) could measure histamine quantities as small as 0 10 μg histamine base per 3 50 ml sample which is sufficient sensitivity as the peritoneal cell suspension contains between 0 5 and 3 μg histamine base per ml (NORN 1968a) The fluorescence intensity was found proportional to a histamine concentration over the range 0 03 to 5 μg per ml and recoveries of histamine added to the cell suspension range from 95 to 100 % (NORN unpublished results) The method must be considered specific for the assay of histamine in rat peritoneal cell suspensions as the histamine content per mast cell has proved to be in complete agreement with results obtained by means of other methods (table 1)

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Duration of histamine release

The anaphylactic release of histamine is a rapid process. Upon incubation of a peritoneal cell suspension with antigen the histamine will be released within 30 sec., i.e. no more histamine will be released upon incubation for 2, 10, or 30 min (NORN 1968a). This result is in keeping with similar experiments using isolated peritoneal mast cells (PERERA & MONGAR 1963b).

Role of other cells

As already mentioned, the peritoneal cell suspension of the rat houses other cells and substances than the mast cells. It seems possible that they may affect the anaphylactic release of histamine from the mast cell, as cells other than the mast cell will be destroyed during the anaphylactic reaction. For instance, polymorphonuclear leukocytes undergo lysis (WAKSMAN 1953), and eosinophilic leukocytes disrupt (GARCIA-AROCHA 1961). This creates the possibility for the release of substances which act upon the mast cells. Indeed, SEEGERS & JANOFF (1966) have demonstrated a mast-cell disrupting factor in polymorphonuclear leukocytes from rabbit exudate. NORN (1968a), therefore investigated whether during anaphylaxis certain cells liberate a factor which in turn releases histamine from the mast cells. The presence of such a factor however was not demonstrable in peritoneal cell suspension after incubation with specific antigen. There is thus every probability that the histamine release from the mast cells is not influenced by other cells or substances in the peritoneal cell suspension. Accordingly, antigen is able to release histamine by reacting only with the mast cell. An isolation of the cell is therefore superfluous. This is essential, because certain isolation or fractionation procedures reduce the sensitivity of the mast cell to antigen, so that mast cells which have been subjected to these procedures cannot be considered biologically intact. This finding has been made in density gradient centrifugation with sucrose (JOHNSON & MORAN 1966) and with bovine serum albumin (NORN 1968a).

Specific antigen-antibody reaction

The release of histamine, caused by antigen, in the peritoneal cell suspension from actively sensitized rats is due to a specific antigen antibody reaction. This has been substantiated by the finding that it is only the specific antigen which releases histamine, while non specific antigens are ineffective (NORN 1967a). The experiment comprised 2 groups of rats, one of which was sensitized to horse serum, and the other to egg albumin. In the former group only horse serum released histamine, and in the latter group only egg

Table 1

Histamine content of the mast cell (μg histamine base per 10^6 cells) determined by various methods

	Biological (guinea pig ileum)	Spectrophotometric (LOWRY et al 1954)	Spectrofluorometric (SHORE et al 1959)	Authors
isolated mast cells from non sensitized rats	16			PERERA & MONGAR 1963
	15–30			UVNAS 1965, UVNAS & THON 1959
	29			LAGUNOFF & BENDITT 1960
non isolated mast cells from non sensitized rats	31			LAGUNOFF & BENDITT 1960
		29		BRAY & ARSDEL 1961
		23		NORN 1965a
non isolated mast cells from sensitized rats		31		NORN 1965a
			21–40	NORN 1968a

Role of the mast-cell concentration

The concentration of mast cells in the peritoneal cell suspension from sensitized rats may vary widely from rat to rat (NORN 1968a) However this is of no importance for the investigation of anaphylactic histamine release *in vitro*, as the same percentage of the total histamine content of the samples *is released at different concentrations of the mast cell* (NORN, 1968a)

Role of antigen concentration

GARCIA-AROCHA (1961) found the antigen concentration to be decisive for the anaphylactic histamine release, the release of histamine taking place only when the concentration of horse serum in the cell suspension was exactly 5 % However, this is in distinct conflict with the results of others *Studies of isolated rat peritoneal mast cells* have shown histamine release at 1 % (UVNAS & THON, 1959) as well as at 20 % horse serum (PERERA & MONGAR, 1963b) NORN (1968a) found, moreover, that anaphylactic histamine release increases in proportion to the logarithm of the antigen concentration

values at pH 6 and pH 9 (rat HOGBERG & UVNAS 1960, guinea pig MONGAR & SCHILD 1958, CHAKRAVARTY 1960) Thus, the curves look like pH activity

from the rat and guinea pig show little degranulation and histamine release (HOGBERG & UVNAS 1960, MONGAR & SCHILD 1958) Similar results are obtained when Ca^{++} is bound by adding EDTA (ethylenediaminetetraacetate) K^{+} and Mg^{++} are of no significance an omission of these cations does not effect the named reactions Nor is Na^{+} decisive, even when sodium chloride is replaced by sucrose, histamine release is still obtained, though reduced (MONGAR & SCHILD 1958) Since several enzymes are activated by Ca^{++}, the role of this cation in the anaphylactic histamine release may hint at the possibility of enzymatic processes as a presupposition of anaphylactic histamine release This hypothesis is supported by the similarity of the temperature and pH curves of the anaphylactic histamine release to corresponding curves for enzymatic processes

The influence of enzyme inhibitors oxygen and glucose on anaphylactic histamine release

To ascertain whether the anaphylactic histamine release is an energy-requiring process the influence of metabolic inhibitors anoxia and glucose has earlier been investigated Inhibitors of oxidative metabolism (dinitrophenol sodium cyanide L thyroxine) inhibit anaphylactic mast cell degranulation and histamine release *in vitro* in samples of mast cells from rats and guinea pigs (HOGBERG & UVNAS 1960, MOTA & ISHII 1960, MONGAR & SCHILD 1957) A similar inhibition is obtained by anoxia induced by nitrogen in glucose free medium in these animal species (DIAMANT 1962a & b, UVNAS 1962 MONGAR & SCHILD 1957) The results indicate the possibility that the anaphylactic histamine release might be an energy requiring process in which the energy is delivered by oxidative metabolism – *i e* oxygen is of primary importance to this release Another possibility is that the inhibition might merely be a consequence of reduced cell function With a view to investigating these possibilities in further detail, the oxygen requirement of the mast cell was measured before and during the anaphylactic reaction Experiments using isolated rat peritoneal mast cell in glucose free medium have given conflicting results By the Cartesian diver technique, CHAKRAVARTY (1968) was able to demonstrate an increase in oxygen uptake immediately after contact with antigen which lasted for 15 to 20 minutes This was not observed by MONGAR & PERERA (1965) using a microcapillary respirometer CHAKRAVARTY s result must be considered correct, because he found a basic oxygen uptake in conformity with the findings of others whereas MONGAR &

Table 2

Release of histamine in peritoneal cell suspensions (PCS) from passively sensitized rats and from non sensitized rats by sera, egg albumin, and modified Tyrode s solution. Duplicate determinations (NORN, unpublished results) For experimental procedure, cf NORN (1967a 1968b)

PCS from rats which were	PCS incubated with	*Released histamine* (per cent of total)
non sensitized	horse serum	3 , 3
	egg albumin	4 5
	mod Tyrode s	3 , 4
sensitized to horse serum	horse serum	29 , 30
	bovine serum	2 , 3
	rat serum	4 , 6
	egg albumin	2 , 4
	mod Tyrode s	3 , 4

albumin In passively sensitized rats the histamine release, too, is due to a specific antigen-antibody reaction (table 2) Let it be emphasized that the spontaneous release of histamine is very slight compared with the anaphylactic histamine release in peritoneal cell suspensions from actively as well as passively sensitized rats (NORN, 1967a, and table 2) Accordingly, the method is well suited for determining anaphylactic release of histamine

The influence of temperature, pH, and ionic environment on anaphylactic histamine release

Anaphylactic mast-cell degranulation and histamine release are dependent upon the temperature Both the degranulation and the histamine release increase from low values at 15° to maximum values in the range 35–42°, for thereafter to fall abruptly to low values around 45° This has been found in the rat as well as in the guinea pig (KELLER & BELGER 1961 CHAKRAVARTY 1960) Strangely enough, HOGBERG & UVNAS (1960) found a wider temperature interval for maximum mast cell disruption in rat mesenterial tissue It is remarkable that these temperature curves are similar to those for enzymatic reactions At 45° and over, the mast cells are influenced so that subsequent incubation at 37° with antigen does not lead to mast-cell degranulation and histamine release This must be due to irreversible injury to a thermolabile factor indispensable in anaphylactic histamine release Moreover, the pH level is decisive to the anaphylactic mast-cell degranulation and histamine release Both are at a maximum at a pH of about 7 5, falling to minimum

Chapter 3

Histamine Liberators

Various substances – *int al* drugs such as polymyxin B, d-tubocurarine, and morphine – *liberate histamine in the body* These histamine liberators have been reviewed by PATON (1957) and GOTH (1967) Administration of histamine liberators elicits the same actions as does histamine, *i e* a fall of arterial blood pressure, shock, and bronchoconstriction (MACINTOSH & PATON 1949, PATON 1951, COLLIER 1956) At the same time, the plasma level of histamine has increased, and in various ways it has been demonstrated that it is histamine – not the histamine liberator – which is the direct trigger of the hypotension (MACINTOSH & PATON 1949, PATON 1951), at least where pure histamine liberators are concerned

The histamine liberators degranulate the mast cells, thereby releasing their content of histamine Thus, compound 48/80 and polymyxin B liberate histamine from most rat tissues (PARRATT & WEST 1957) UVNAS and his colleagues assume that liberators such as compound 48/80, lecithinase A (phosphatidase A or phospholipase A) and α-chymotrypsin liberate histamine from rat mast cells by eliciting the same enzymatic and energy requiring mechanism in the mast cell as does the antigen-antibody reaction This hypothesis is indirectly supported by the fact that histamine release and mast cell degranulation, caused by these histamine liberators and by the antigen-antibody reaction, are influenced in the same way by factors such as temperature, pH, ionic environment, enzyme inhibitors, anoxia, and glucose (HOGBERG & UVNAS 1960, DIAMANT 1962, UVNAS 1962 & 1963, UVNAS & ANTONSSON 1963) If this hypothesis is correct, these histamine liberators are well-suited tools for studying separately the biochemical mechanism of the anaphylactic histamine release However, the present study shows that the hypothesis is not quite correct. NORN (1965a & 1967b) investigated the histamine release in peritoneal cell suspensions from the rat in the antigen-antibody reaction as well as with the named histamine liberators These studies revealed that in cases where rats have been pre-treated by the same antirheumatic drug, *e g* sodium aurothiosulphate or phenylbutazone, the histamine release caused by the histamine liberators is nevertheless influenced in different ways (table 3) This shows that the histamine liberators examined must liberate histamine by eliciting mutually different biochemical mechanisms The next problem is then whether one of these histamine

PERERA did not find this (CHAKRAVARTY 1968) The increase in oxygen requirement *by the anaphylactic reaction supports the hypothesis that* anaphylactic histamine release is an energy-requiring process, in which the energy is supplied by oxidative metabolism However, the energy can also be supplied by glycolysis – at least during anoxia Thus, the inhibitory effect of anoxia upon histamine release is completely abolished when mast-cell samples from guinea-pigs and rats contain glucose (DIAMANT 1962a & b, UVNÄS 1962) The influence upon the anaphylactic histamine release by pH, temperature, Ca^{++}, metabolic inhibitors, anoxia, and glucose, thus indicates that the release is due to enzymatic and energy-requiring processes As already mentioned, these actions give rise to the same response in mast-cell samples from the rat and from the guinea-pig Therefore, the biochemical mechanism in the two animal species seems to be the same in all essentials That minor differences exist will, however, appear from the following facts *1*) the serine esterase inhibitors phenylalkyl- and chloroalkyl phosphonates show inhibition profiles in anaphylactic histamine release which differ for the two animal species (BECKER & AUSTEN 1964 & 1966, BECKER 1968), and 2) 2-deoxyglucose influences the anaphylactic histamine release differently in the rat and in the guinea-pig (CHAKRAVARTY 1962 & 1968b)

In the present study anaphylactic histamine release was investigated in rat peritoneal cell suspensions to which no glucose had been added In order to ascertain whether the energy conditions were sufficient, i e if the histamine release was equal to that in samples with glucose, samples with glucose and samples without glucose from test and control rats in the various experiments (table 7) were compared The release of histamine was the same in both cases, so that the energy conditions must have been sufficient

oxygen uptake during histamine release elicited by compound 48/80 in glucose free medium, even though such an increase takes place during anaphylactic histamine release (p 25)

On the basis of the above findings by CHAKRAVARTY, KAHL & NETTER and NORN it must be concluded that histamine liberators cannot be used as tools for investigating the biochemical mechanism in the anaphylactic release of histamine

Table 3.

Inhibitory effect of antirheumatic agents administered to the rat on the release of histamine from their peritoneal cells by histamine liberators (NORN 1967b) and by antigen (NORN 1965a)

Pre treatment *for 3 days with* antirheumatic *agents*	Inhibitory effect of antirheumatic agents in % on histamine release in control group after				
	Compound 48/80 0 125 μg/ml	Polymyxin B 0 50 μg/ml	Lecithinase A 1 50 μg/ml	α Chymotrypsin 100 μg/ml	Antigen
Hydrocortisone 100 mg/kg	6	0	0	0	58**
Phenylbutazone 200 mg/kg	0	0	15*, 26*	0	32*
Sodium aurothio sulphate 25 mg/kg	8, 19	18	22*, 28*	70**	87**

* $p < 0\ 05$ ** $p < 0\ 01$ by Student's t test

liberators liberates histamine by the same biochemical mechanism as that *of the antigen-antibody reaction* *This cannot be decided from these experiments* (table 3), which appears from the following facts 1) The finding that an antirheumatic agent inhibits the histamine release by the anaphylactic reaction, but not by the histamine liberator, can be explained in at least two ways either the enzymatic processes elicited in the anaphylactic reaction are different from those elicited by the histamine liberator, or the antirheumatic agent does not influence the enzymatic processes during the anaphylactic reaction, whereas it influences the antigen-antibody binding 2) The opposite finding, viz that an antirheumatic agent inhibits the histamine release by the anaphylactic reaction as well as by the histamine liberator, gives at least two possibilities, namely either inhibition of the same enzymatic processes, or inhibition of two different enzymatic processes, *which both could* be inhibited by the same antirheumatic agent

As regards compound 48/80 it has, however, been established that this histamine liberator does not release histamine by the same biochemical mechanism as that of the antigen-antibody reaction This will appear from the following findings in the rat 1) CHAKRAVARTY (1968b) found the anaphylactic histamine release to be inhibited by 2-deoxyglucose in aerobic, glucose-free medium, while this did not apply to the histamine release by compound 48/80, and 2) KAHL & NETTER (1967) found no increase in

Table 4

Inhibitory effect of antirheumatic agents on anaphylactic histamine release from guinea pig lung *in vitro* The lungs were pre incubated with antirheumatic agent and thereafter incubated with antigen. The inhibition in anaphylactic histamine release is given corresponding to the concentration of antirheumatic agent (µg/ml perfusion solution or lung suspension)

	Percentage inhibition				
Antirheumatic agent	Perfused lung		Chopped lung		Author
Cortisone	40 and 100	0			Trethewie 1958
Phenylbutazone	25, 50 and 100	100			Trethewie 1957
			1000	50	Mongar & Schild 1957
Sodium salicylate	500 and 1000	100			Trethewie 1951
			10 000	50	Mongar & Schild 1957
Acetylsalicylic acid	500 and 1000	100			Trethewie 1951
Amidopyrine			2000	50	Mongar & Schild 1957

sulphate The finding that phenylbutazone was more potent than amidopyrine is in agreement with the results obtained by Mota & Ishii (1960)

It appears from these *in vitro* experiments that there is *no* definite difference between the way in which non steroid antirheumatics influence anaphylactic histamine release from the rat and the way in which they influence anaphylactic histamine release from the guinea pig As regards steroid antirheumatics, there *is* a definite difference, as glucocorticoids inhibit anaphylactic histamine release from the rat, but not from the guinea pig Recalling the three main phases in anaphylactic histamine release (p 17) the conclusion can be drawn that the mechanism of action for the inhibitory effect of these antirheumatics must be due exclusively to inhibition of the antigen antibody reaction, of the biochemical mechanism or both, – since the experiments were based on sensitized mast cells with the antibody already bound to the cell

(B) *In vivo-in vitro experiments*

Such experiments seem to have been carried out only by Goadby & Smith (1964) and by Norn Goadby & Smith (1964) treated sensitized guinea pigs with 50 mg/animal of hydrocortisone sodium hemisuccinate intramuscularly Eighteen hours later the lungs were isolated and perfused with antigen. The treatment with glucocorticoid did not inhibit the ana-

Chapter 4

Influence of Antirheumatics on Anaphylactic Histamine Release

The influence of antirheumatic agents on anaphylactic histamine release has been studied by (A) *in vitro* experiments and (B) *in vivo-in vitro* experiments In (A), tissue or peritoneal cells from actively sensitized animals were pre-incubated with antirheumatic agents prior to incubation with antigen In (B), actively sensitized animals were given antirheumatics and were then killed, whereafter tissue or peritoneal cells were isolated and incubated with antigen *in vitro*

(A) *In vitro experiments*

In the 1950 s TRETHEWIE found that sodium salicylate, acetylsalicylic acid, and phenylbutazone inhibited the anaphylactic histamine release from isolated and perfused guinea-pig lungs (TRETHEWIE 1951 and 1957), while cortisone was ineffective (TRETHEWIE 1958) (table 4) The effect of non-steroid antirheumatics was later confirmed by MONGAR & SCHILD (1957) in experiments on chopped guinea-pig lung tissue (table 4) From the guinea-pig experiments it can be concluded that non-steroid antirheumatics are able to inhibit anaphylactic histamine release, while glucocorticoids are without effect Among the non-steroid antirheumatics, phenylbutazone is more potent than either sodium salicylate, acetylsalicylic acid, or amidopyrine

Experiments on rat tissue were made by MOTA & ISHII and later by NORN MOTA & ISHII (1960) found that phenylbutazone and amidopyrine inhibited anaphylactic histamine release in suspensions of cutaneous tissue Phenylbutazone was more potent than amidopyrine An inhibition of 45 % was obtained by phenylbutazone and amidopyrine in concentrations of, respectively, 1540 and 2300 μg/ml NORN (1965a, and unpublished results) has examined steroid- and non-steroid antirheumatics in suspensions of peritoneal cells (table 5) These experiments showed that both steroid antirheumatics and non-steroid antirheumatics were able to inhibit anaphylactic histamine release from rat mast cells *in vitro* Hydrocortisone is the most potent antirheumatic agent, then follow phenylbutazone, and the least potent agents are sodium salicylate, sodium-γ-resorcylate, and amidopyrine No inhibitory effect could be found by means of sodium aurothio-

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Inhibitory effect of antirheumatic agents on anaphylactic histamine release from guinea pig lung *in vitro* The lungs were pre-incubated with antirheumatic agent and thereafter incubated with antigen The inhibition in anaphylactic histamine release is given corresponding to the concentration of antirheumatic agent (µg/ml perfusion solution or lung suspension)

	Percentage inhibition				
Antirheumatic agent	Perfused lung		Chopped lung		Author
Cortisone	40 and 100	0			Trethewie 1958
Phenylbutazone	25 50 and 100	100			Trethewie 1957
			1000	50	Mongar & Schild 1957
Sodium salicylate	500 and 1000	100			Trethewie 1951
			10 000	50	Mongar & Schild 1957
Acetylsalicyl c acid	500 and 1000	100			Trethewie 1951
Amidopyrine			2000	50	Mongar & Schild 1957

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It appears from these *in vitro* experiments that there is *no* definite difference between the way in which non steroid antirheumatics influence anaphylactic histamine release from the rat and the way in which they influence anaphylactic histamine release from the guinea pig As regards steroid antirheumatics, there *is* a definite difference, as glucocorticoids inhibit anaphylactic histamine release from the rat, but not from the guinea pig Recalling the three main phases in anaphylactic histamine release (p 17) the conclusion can be drawn that the mechanism of action for the inhibitory effect of these antirheumatics must be due exclusively to inhibition of the antigen antibody reaction, of the biochemical mechanism or both – since the experiments were based on sensitized mast cells with the antibody already bound to the cell

(B) *In vivo-in vitro experiments*

Such experiments seem to have been carried out only by Goadby & Smith (1964) and by Norn Goadby & Smith (1964) treated sensitized guinea pigs with 50 mg/animal of hydrocortisone sodium hemisuccinate intramuscularly Eighteen hours later the lungs were isolated and perfused with antigen The treatment with glucocorticoid did not inhibit the ana

phylactic histamine release from the lungs, since the yield of released histamine was the same as that in the control group

In a preliminary study (NORN 1965a), NORN has investigated the influence of several steroid- and non-steroid antirheumatics on anaphylactic histamine release from the rat Male albino rats were actively sensitized to horse serum During the last 3 days of the sensitization period (21 days) half of the rats were given a daily subcutaneous injection with an antirheumatic agent 1½–2 hrs after the last treatment with antirheumatic agent the rats were killed, and the peritoneal cavities were washed in order to obtain peritoneal cells The cell suspensions were incubated with antigen *in vitro*, and the quantity of released histamine was then determined as the percentage of the total histamine content of the sample The results appear from table 6 Only clinically potent antirheumatic agents showed significant inhibition of the anaphylactic histamine release, i e all the glucocorticoids studied, and among non-steroids only sodium aurothiosulphate and phenylbutazone Without any effect were monophenylbutazone, oxiphenbutazone, indomethacin, sodium salicylate, sodium-γ-resorcylate, acetylsalicylic acid and amidopyrine As to the glucocorticoids, their relative activities (ED_{50} doses, equal to a 50 % inhibition of histamine release) corresponded fairly well with their clinically anti-inflammatory potency Sodium aurothiosulphate was more potent than hydrocortisone, while phenylbutazone was less potent A very low dose of indomethacin was administered, because a

Table 5

Inhibitory effect of antirheumatic agents on the anaphylactic histamine release in suspensions of peritoneal cells from rats The cell suspensions were pre incubated with antirheumatic agent for 30 min and thereafter incubated with antigen for 30 min In both instances the temperature was 38° The results represent the mean value of 3 samples (NORN, unpublished results) Exactly the same results were obtained when the antirheumatic agent was added just before the incubation with antigen (NORN 1965a)

Antirheumatic agent	Percentage inhibition of histamine release — Added µg substance per ml peritoneal cell suspension						
	0 1	1	10	50	100	1000	10000
Hydrocortisone	0	0	0	26	93		
Phenylbutazone	0	0	0		27	95	
Sodium aurothiosulphate	0	0	0		0	0	
Sodium salicylate	0	0	0		3	74	98
Sodium γ resorcylate	0	0	2		16	11	77
Amidopyrine	0	0	0		0	6	95

higher dose would have resulted in development of multiple intestinal necroses and peritonitis Throughout the experiments the inhibition was due to a reduced liberation of intracellular histamine from the mast cells This is a consequence of the fact that in the peritoneal cell suspension,

Table 6

Inhibitory effect of antirheumatic agents on the anaphylactic histamine release in peritoneal cell suspensions from actively sensitized rats. The rats were treated during the last 3 days of the sensitization period with a daily subcutaneous injection of antirheumatic agent. Suspensions of peritoneal cells were then removed and incubated *in vitro* with antigen. Percentage inhibition of the anaphylactic histamine release is given (NORN 1965a)

Inhibition of histamine release					
Test specimens number	Control specimens number	Substance	Daily dose 3 days mg/kg	% inhibition	P
4	3	Hydrocortisone	200	60	+
14	11	Hydrocortisone	100	58	++
4	3	Hydrocortisone	50	43	
4	3	Hydrocortisone	25	18	
5	5	Fluorometholone	100	69	+
5	5	Fluorometholone	50	39	
5	5	Fluorometholone	25	7	
10	10	Methylprednisolone	5 0	75	++
5	5	Methylprednisolone	3 6	33	
5	5	Methylprednisolone	2.5	14	
5	5	Betamethasone	5 0	82	++
5	5	Betamethasone	2.5	37	
5	5	Betamethasone	1 25	34	
5	5	Betamethasone	0 63	0	
15	14	Phenylbutazone	200	32	+
4	4	Phenylbutazone	100	20	
5	5	Monophenylbutazone	400	0	
5	5	Monophenylbutazone	100	2	
5	5	Oxiphenbutazone	200	0	
5	5	Sodium aurothio-	25	87	++
5	5	sulphate	5	0	
5	5	Indomethacin	2.5	0	
4	4	Sodium salicylate	200	0	
4	4	Sodium γ resorcylate	200	0	
5	5	Acetylsalicylic acid	150	0	
8	8	Amidopyrine	200	14	

+ $p < 0.05$ ++ $p < 0.01$ by Student's t test.

Table 7.

Influence of antirheumatic agents on anaphylactic histamine release from peritoneal cells of actively and passively sensitized rats Actively sensitized rats (*donors*) were given antirheumatic agents, and serum from these rats transferred to non sensitized rats (*recipients*) Suspensions of peritoneal cells from donors and recipients were then incubated with antigen Each group comprises 8–12 rats, and the figures represent mean values and standard errors of the mean (NORN 1968b & 1969) No glucose had been added to the peritoneal cell suspensions (cf p 26) Samples of peritoneal cell suspensions with added glucose (2 mg per ml) from test and control rats in all experiments gave, however, the same results

Treatment of donors, daily dose	% histamine release by Donors Control	Donors Test	Recipients Control	Recipients Test
(1) *during the last 3 days of the sensitization period*				
Hydrocortisone 100 mg/kg	46 ± 4	15 ± 4**	73 ± 6	74 ± 7
Sodium aurothiosulphate 25 mg/kg	41 ± 5	17 ± 3**	49 ± 7	39 ± 6
Phenylbutazone 200 mg/kg	68 ± 5	40 ± 4**	34 ± 7	32 ± 8
(2) *during the first week of the sensitization period*				
Hydrocortisone 43 mg/kg	57 ± 6	34 ± 8*	50 ± 9	18 ± 5**
21 mg/kg	52 ± 5	45 ± 6	60 ± 5	25 ± 5**
Sodium aurothiosulphate 21 mg/kg	52 ± 5	44 ± 4	60 ± 5	56 ± 7
Phenylbutazone 86 mg/kg	57 ± 6	49 ± 4	50 ± 9	40 ± 5
(3) *during 3 weeks previous to the sensitization period*				
Hydrocortisone 17 mg/kg	50 ± 7	55 ± 5	30 ± 6	31 ± 5
12 mg/kg	61 ± 7	58 ± 4	16 ± 4	20 ± 4
Sodium aurothiosulphate 17 mg/kg	69 ± 4	69 ± 5	35 ± 7	30 ± 6
Phenylbutazone 67 mg/kg	61 ± 7	69 ± 3	16 ± 4	16 ± 3
67 mg/kg	50 ± 7	52 ± 6	30 ± 6	26 ± 5

(1) the rats were killed 1 5–2 hrs after the last administration of antirheumatic agent
(2) the first dose of antirheumatics was given 1 hr before the first administration of antigen
(3) the last dose of antirheumatics was given 48 hrs before the first administration of antigen
* $p < 0.05$ ** $p < 0.01$ by Student's t test.

the concentration of histamine and the concentration of mast cells had not been altered, – nor had the spontaneous histamine release been affected (NORN 1965a)

The preliminary study on rats (NORN 1965a) was thereafter extended by NORN with a view to investigating the effect of the antirheumatic agents when given at different times of the sensitization period (1) towards the end, (2) at the beginning, and (3) prior to the sensitization period In addition, the mechanism of action was studied These extended investigations comprised only antirheumatic agents which had proved effective in the preliminary study, i e glucocorticoids (hydrocortisone), sodium aurothiosulphate and phenylbutazone All these experiments were carried out as mentioned for the preliminary study, with the exceptions that female rats were used instead of males, and fluorometric determination of histamine (NORN 1967b) instead of spectrophotometric determination The results are summarized in table 7

(1) *Treatment towards the end of the sensitization period, i e* daily subcutaneous administration during the last 3 days of the sensitization period The experiment is identical with that of the preliminary study (NORN 1965a) with the exceptions mentioned above Hydrocortisone, sodium aurothiosulphate and phenylbutazone showed significant inhibition of the anaphylactic histamine release (NORN 1969) as had been the case in the preliminary study The results also appear from table 7 (1), *the donors* The treatment with hydrocortisone and sodium aurothiosulphate did not appear to affect the general condition of the rats, but on the other hand, 2 out of 10 rats died after the administration of phenylbutazone (NORN 1968b) With a view to investigating the mechanism of action, the antibody production was assessed in terms of concentration of mast cell-sensitizing antibody in the serum Since this concentration cannot be determined directly by precipitation of the antibody in the serum (MOTA 1963c and 1964, BINAGHI *et al* 1964), it was measured indirectly by the ability of serum to elicit passive sensitization in non-sensitized rats (recipients) The recipients were therefore used only as an antibody assay system Serum from each donor, both test and control animals, was dialyzed to remove any content of antirheumatic substance, thereafter it was diluted with Tyrode solution, and then injected intraperitoneally into a recipient (NORN 1968b) The degree of the passive sensitization was measured 48 hrs later as the release of histamine in the peritoneal cell suspension of the recipient after incubation with antigen *in vitro* (NORN 1968b) The results appear from table 7, (1) *the recipients*, and from NORN (1968b) The histamine release by the recipients was the same whether they received serum from untreated donors or from donors treated with hydrocortisone, sodium aurothiosulphate or phenylbutazone Therefore, treatment of the donors with these agents does not alter the concentration of mast cell-sensitizing antibody in their serum. Consequently the inhibitory effect of the antirheumatic agents on the anaphylactic histamine release by the donors cannot be due

Table 7.

Influence of antirheumatic agents on anaphylactic histamine release from peritoneal cells of actively and passively sensitized rats Actively sensitized rats (donors) were given antirheumatic agents, and serum from these rats transferred to non sensitized rats (recipients) Suspensions of peritoneal cells from donors and recipients were then incubated with antigen Each group comprises 8–12 rats, and the figures represent mean values and standard errors of the mean (NORN 1968b & 1969) No glucose had been added to the peritoneal cell suspensions (cf p 26) Samples of peritoneal cell suspensions with added glucose (2 mg per ml) from test and control rats in all experiments gave, however, the same results

Treatment of donors, daily dose	% histamine release by			
	Donors		Recipients	
	Control	Test	Control	Test
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(1) the rats were killed 1 5–2 hrs after the last administration of antirheumatic agent
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* $p < 0\ 05$ ** $p < 0\ 01$ by Student's t test

the concentration of histamine and the concentration of mast cells had *not been altered*, – nor had the spontaneous histamine release been affected (NORN 1965a)

in the sensitization period, when they weighed approx 20–40 g less than the control animals The treatment with hydrocortisone resulted in a reduced concentration of mast cell-sensitizing antibody *in the serum* This inhibition of the antibody production explains the mechanism involved in the inhibitory effect of hydrocortisone upon the anaphylactic histamine release in the present experiment It cannot be ruled out, however, that the other two possibilities, viz inhibition of the antigen-antibody reaction and inhibition of the biochemical mechanism, may have been contributory

(3) *Treatment prior to the sensitization period, i e* daily subcutaneous administration during 3 weeks prior to the sensitization period Treatment with hydrocortisone, phenylbutazone and sodium aurothiosulphate did not alter the anaphylactic histamine release at all (table 7 (3), and NORN (1969)) Throughout the investigation, the treatment did not alter the concentration of histamine in the peritoneal cell suspension, nor did it affect the spontaneous histamine release The general condition of the rats was not affected, apart from the fact that the rats, treated with hydrocortisone and sodium aurothiosulphate, gained less in weight than the control rats

From the *in vivo-in vitro* experiments on rats (tables 6 and 7) it can be concluded that treatment of rats with antirheumatic agents in reasonable dosage can inhibit anaphylactic histamine release Only clinically potent antirheumatics glucocorticoids as well as non steroids (sodium aurothiosulphate and phenylbutazone), inhibited histamine release Throughout the experiments, the inhibition was due to a reduced liberation of intracellular histamine from the mast cells Hydrocortisone inhibited anaphylactic histamine release when given at the beginning or at the end of the sensitization period The inhibition is due to different mechanisms, depending on the time of the sensitization period at which the agent is administered On administration at the beginning of the period, the mechanism will depend on inhibition of the formation of mast cell sensitizing antibody, but on administration towards the end of the sensitization period, it will depend on inhibition of the antigen antibody reaction, or on inhibition of the biochemical mechanism, or possibly on both Only when given towards the end of the sensitization period will phenylbutazone and sodium aurothiosulphate inhibit the anaphylactic histamine release The mechanism is an inhibition of the antigen antibody reaction, of the biochemical mechanism, – or possibly of both

Influence of antirheumatic agents upon the formation of mast cell-sensitizing antibody has not been reported previously On the other hand, it has long been known that the *production* of various *circulating antibodies* can be suppressed in most animal species by means of administration of certain antirheumatic agents during the sensitization period This is most marked for glucocorticoids (GERMUTH & OTTINGER 1950, GERMUTH *et al*

to inhibition of the antibody production In accordance with the three main phases mentioned on p 17, the mechanism must therefore be due to inhibition of either the antigen-antibody reaction, or to inhibition of the biochemical mechanism, or possibly both Direct proof of such a mechanism (or mechanisms) was examined only in the case of hydrocortisone (table 8, and NORN 1968b) Rats were passively sensitized with pooled serum from actively sensitized rats Half of the recipients were pre-treated with a daily subcutaneous injection of hydrocortisone, starting before, or at the same time as, the passive sensitization 48 hrs after the passive sensitization, peritoneal cells from each of the recipients were incubated with antigen Groups, pre-treated with hydrocortisone, showed a significant decrease in the release of histamine This indicates directly that hydrocortisone can inhibit the antigen-antibody reaction, the biochemical mechanism, or both It was not possible to distinguish between these possibilities, as histamine liberators are not suitable tools in the study of the biochemical mechanism (chapter 3)

(2) *Treatment at the beginning of the sensitization period, i e* daily subcutaneous administration during the first week of the sensitization period The treatment caused a significant inhibition of the anaphylactic histamine release by means of hydrocortisone, while sodium aurothiosulphate and phenylbutazone proved ineffective (table 7 (2), and NORN 1969) Throughout the investigation, the treatment did not alter the concentration of histamine in the peritoneal cell suspension, nor did it affect the spontaneous histamine release The doses administered did not appear to affect the general condition of the rats, apart from the fact that the rats, treated with hydrocortisone and sodium aurothiosulphate, gained less weight, especially early

Table 8

Influence of hydrocortisone treatment of passively sensitized rats on the anaphylactic histamine release from their peritoneal cells Each group comprises eight animals and the figures represent the mean values and the standard error of the mean (NORN 1968b)

Hydrocortisone treatment	Histamine release in per cent		% decrease
	Control	Test	
6 days, 50 mg/kg daily starting 3 days before the passive sensitization	51 ± 7 43 ± 6	21 ± 6** 7 ± 2**	59 84
3 days, 100 mg/kg daily starting at the same time as the passive sensitization	73 ± 3	53 ± 5**	27

** $p < 0\ 01$ by Student's t test

experiments, with the exception that sodium aurothiosulphate does not act *in vitro*

As regards the mechanism of action, it is possible by means of the *in vivo-in vitro* experiments to examine whether antirheumatic agents can inhibit the anaphylactic histamine release by (a) an inhibited antibody formation, or (b) an inhibition of the antigen antibody reaction, or/and an inhibition of the biochemical mechanism In contrast to this, the *in vitro* experiments can only reveal a mechanism depending on (b), because the experiments are based on sensitized mast cells with the antibody already bound to the cell

1952, GERMUTH 1956, RAFFEL 1961), less pronounced for salicylates (SWIFT 1922, SMULL *et al* 1948, AUSTEN 1963) The effect of phenylbutazone has not yet been sufficiently elucidated, but experiments on the rabbit indicate that the antibody production is practically unaffected (ROTHER & KATZ 1957, RECHENBERG 1962) One study on gold (PERSELLIN *et al* 1967) showed no change in antibody production in the rabbit The conclusion may be drawn, that since hydrocortisone inhibits the production of both mast cell-sensitizing antibody and the production of other circulating antibodies, and since phenylbutazone as well as gold have proved ineffective on either, there is possibly a parallelism between the influence of anti-rheumatic agents upon the production of mast cell-sensitizing antibody and their influence on the production of other circulating antibodies

A comparison between *the rat and the guinea-pig* as *regards the influence* of antirheumatics upon anaphylactic histamine release in the *in vivo-in vitro* experiments is only possible in the case of glucocorticoids, since non-steroids have not been examined in the guinea-pig The fact that hydrocortisone inhibits histamine release from the rat, but not from the guinea pig, shows that in the case of steroid antirheumatics there is a definite difference between these animal species

Comparison between in vitro experiments and in vivo in vitro experiments

As regards the influence of antirheumatics upon anaphylactic histamine release in the rat, there is a certain agreement between the *in vitro* experiments and the *in vivo-in vitro* experiments (table 5, 6, and 7 (1)) The finding that hydrocortisone is more potent than phenylbutazone, and that sodium salicylate, sodium-γ-resorcylate and amidopyrine are less potent than phenyl butazone showed agreement In very high concentrations, sodium salicylate sodium-γ-resorcylate and amidopyrine inhibited the anaphylactic histamine release in the *in vitro* experiments Such high concentrations cannot be obtained *in vivo* with reasonable doses No inhibition was therefore obtained by means of the *in vivo-in vitro* experiments The discrepancy appears from the fact that sodium aurothiosulphate inhibits histamine release in *in vivo-in vitro* experiments, but it is completely without effect *in vitro* Perhaps the effect of sodium aurothiosulphate is obtained only *in vivo* because of a time-requiring process, – perhaps the cause may be found in connection with a reduction to metallic gold *It is remarkable that in the in vivo in vitro* experiments, sodium aurothiosulphate is more potent than hydrocortisone From these comparisons it appears that only clinically potent antirheumatics are active in the *in vivo-in vitro* experiments, whereas in the *in vitro* experiments an effect is also obtained with weaker antirheumatics, *viz* salicylates and amidopyrine The order of potency is the same in both sorts of

release In the *in vivo-in vitro* experiments (tables 6 and 7) only clinically potent antirheumatics, i e. glucocorticoids, sodium aurothiosulphate and phenylbutazone, acted In the *in vitro* experiments (*table 5*) an effect was also obtained with weaker anti-rheumatics, i e sodium salicylate, sodium γ-resorcylate and amidopyrine, in high concentrations, which cannot be obtained *in vivo* with reasonable doses Except for sodium aurothiosulphate which did not act *in vitro*, the order of potency was the same (sodium aurothiosulphate > hydrocortisone > phenylbutazone > salicylate and amidopyrine) There was thus a partial agreement between the effect of individual antirheumatic agents in the *in vivo-in vitro* experiments and that in the *in vitro* experiments With respect to the glucocorticoids examined by means of the *in* [illegible] ...periments the relative activities (ED_{30} doses, corresponding [illegible]
with their [illegible]

[illegible] *in vitro* experiments (table 7) the inhibitory effect was due

[illegible]

hibited the anaphylactic histamine release when given at the beginning [illegible] towards the end of the sensitization period During the initial phase, the mechanism depended on inhibition of the formation of mast cell-sensitizing antibody During the late phase it was due to inhibition of the antigen antibody reaction or inhibition of the biochemical mechanism, or of both Phenylbutazone and sodium aurothiosulphate inhibited the anaphylactic histamine release only when given towards the end of the sensitization period. The mechanism is an inhibition of the antigen-antibody reaction, of the biochemical mechanism or of both Administration of hydrocortisone, phenylbutazone and sodium aurothiosulphate prior to the sensitization period does not influence the histamine release

In the in vitro experiments (*table 5*) hydrocortisone, phenylbutazone, sodium salicylate, sodium-γ-resorcylate and amidopyrine inhibited the anaphylactic histamine release by a mechanism depending on inhibition of the antigen-antibody reaction, of the biochemical mechanism or of both

In accordance with the results mentioned above, it is possible by means of *in vivo-in vitro* experiments to distinguish between the mechanisms (a), an inhibition of the antibody formation, and (b), an inhibition of the antigen-antibody reaction, or/and an inhibition of the biochemical mechanism In contrast to this, the *in vitro experiments* can only reveal a mechanism depending on (b) An attempt to distinguish between the antigen-antibody reaction and the biochemical mechanism by means of the histamine liberators, compound 48/80, polymyxin B, lecithinase A, and α-chymotrypsin, revealed that this was not possible (cf. chapter 3, and NORN 1967b) The experiment (NORN 1967b) thus indicated that histamine liberators release histamine by

Chapter 5

Conclusions and Perspectives

On the basis of the preceding chapters and the author's previous results, an attempt will now be made to elucidate the problems posed in the introduction (cf p 14)

Re item 1

The developed method meets the demands to be made on the assessment of anaphylactic histamine release, since

(a) the determination of histamine in the cell suspension is specific and sufficiently sensitive (cf p 20 and 21, NORN 1965b & 1967b, SHORE *et al* 1959),

(b) histamine release has been shown by this method to depend upon a specific antigen antibody reaction (cf p 23, and NORN 1967a),

(c) the released histamine originates almost exclusively from the mast cells, and the histamine release is not affected by the other cells or substances in the suspension, thus, isolation of mast cells, which might lead to a change in their sensitivity to antigen, is avoided (cf p 21 and 23, NORN 1968a),

(d) anaphylactic histamine release is high, and spontaneous histamine release slight (cf p 24, NORN 1967a),

(e) the mast-cell concentration in the cell suspension can be determined (cf p 20)

Re item 2

For anaphylactic histamine release we found a relationship between the antigen concentration and the histamine release Thus, the anaphylactic histamine release increases in proportion to the logarithm of the antigen concentration (p 22) Furthermore it is established that anaphylactic histamine release is a rapid process (p 23)

The influence of antirheumatic agents upon the anaphylactic histamine release has been studied on the rat (NORN 1965a and 1969) by *in vivo in vitro* experiments and by *in vitro* experiments In both types of experiments steroids as well as non steroid antirheumatics were able to inhibit histamine

release In the *in vivo-in vitro* experiments (tables 6 and 7) only clinically potent antirheumatics, i e glucocorticoids, sodium aurothiosulphate and phenylbutazone, acted In the *in vitro* experiments (table 5) an effect was also obtained with weaker anti-rheumatics, i e sodium salicylate, sodium-γ-resorcylate and amidopyrine, in high concentrations, which cannot be obtained *in vivo* with reasonable doses Except for sodium aurothiosulphate which did not act *in vitro*, the order of potency was the same (sodium aurothiosulphate > hydrocortisone > phenylbutazone > salicylate and amidopyrine) There was thus a partial agreement between the effect of individual antirheumatic agents in the *in vivo-in vitro* experiments and that in the *in vitro* experiments With respect to the glucocorticoids examined by means of the *in vivo-in* [illegible] experiments the relative activities (ED_{50} doses, corresponding to a [illegible]
with their clinical [illegible]

In the *in vivo in vitro* experiments (table 7) the inhibitory effect was due to different mechanisms, depending on the time of the sensitization period at which the anti rheumatic agent was administered Thus, hydrocortisone inhibited the anaphylactic histamine release when given at the beginning or towards the end of the sensitization period During the initial phase, the mechanism depended on inhibition of the formation of mast cell-sensitizing antibody During the late phase it was due to inhibition of the antigen antibody reaction or inhibition of the biochemical mechanism, or of both Phenylbutazone and sodium aurothiosulphate inhibited the anaphylactic histamine release only when given towards the end of the sensitization period The mechanism is an inhibition of the antigen antibody reaction, of the biochemical mechanism or of both Administration of hydrocortisone, phenylbutazone and sodium aurothiosulphate prior to the sensitization period does not influence the histamine release

In the in vitro experiments (table 5) hydrocortisone, phenylbutazone sodium salicylate, sodium-γ-resorcylate and amidopyrine inhibited the anaphylactic histamine release by a mechanism depending on inhibition of the antigen antibody reaction of the biochemical mechanism or of both

In accordance with the results mentioned above, it is possible by means of *in vivo-in vitro* experiments to distinguish between the mechanisms (a), an inhibition of the antibody formation, and (b), an inhibition of the antigen-antibody reaction, or/and an inhibition of the biochemical mechanism In contrast to this, the *in vitro* experiments can only reveal a mechanism depending on (b) An attempt to distinguish between the antigen antibody reaction and the biochemical mechanism by means of the histamine liberators, compound 48/80, polymyxin B lecithinase A, and α-chymotrypsin, revealed that this was not possible (cf chapter 3, and NORN 1967b) The experiment (NORN 1967b) thus indicated that histamine liberators release histamine by

means of mutually different biochemical mechanisms It also showed that it is questionable whether one of them will release histamine by the same biochemical mechanism as that triggered off by the antigen antibody reaction (chapter 3)

The present study was based exclusively on rat experiments To elucidate the possibility of species differences in respect to anaphylactic histamine release, results obtained by other authors in the guinea pig have been quoted Table 9 gives a list of differences and similarities between the anaphylactic reaction patterns in these two species Items 1–4 in the table demonstrate some

Table 9

Species differences in anaphylaxis in the rat and guinea pig

		Rat	Guinea pig	Page
1	Antihistamines counteract non fatal A S	+	+	14
2	Antihistamines protect from fatal A S	−	+	14
3	Sensitivity to administered histamine	slight	high	14
4	Cortisone protects from fatal A S	+	−	14
5	Marked anaphylactic hypersensitivity is obtained by			
	antigen alone	−	+	17
	antigen together with special adjuvant	+		17
6	The antibody responsible for the anaphylaxis			
	is precipitating	−	+	18 19
	is heat stable at 56°	−	+	19
7	Temperature and pH curves for A H R are equal to temperature and pH curves for enzymatic processes	+	+	24
8	Ca^{++} is necessary for A H R	+	+	25
9	Inhibitors of oxidative metabolism inhibit A H R	+	+	25
10	Anoxia inhibits A H R	+	+	25
11	*During anoxia A H R is re established by glucose*	+	+	26
12	Serine esterase inhibitors give rise to an inhibition pattern in A H R *different* in the rat and in the guinea pig			26
13	2 deoxyglucose affects A H R *differently* in the rat and in the guinea pig			26
14	Glucocorticoid *in vivo* inhibits A H R.	+		31 32
15	Glucocorticoid *in vitro* inhibits A H R	+	−	30 31
16	Phenylbutazone *in vitro* inhibits A H R	+	+	30 31
17	Sodium salicylate *in vitro* inhibits A H R	+	+	30 31
18	Amidopyrine *in vitro* inhibits A H R	+	+	30 31

A S = anaphylactic shock
A H R = anaphylactic histamine release
\+ yes − no

differences However, these differences are not necessarily due to differences in the mechanism of histamine release, which is the subject of the present discussion, since these items concern anaphylactic reaction in general The differences might therefore be dependent on other factors than histamine for contribution to the anaphylactic reaction (p 14) *Immunologically*, there are definite differences between the two species (items 5 and 6) These differences may, however, be of little or no importance to the influence of antirheumatics upon anaphylactic histamine release, since there appears to be agreement between the effect of these agents upon the formation of mast cell sensitizing antibody and the formation of other circulating antibodies (p 38) In all essentials the *biochemical mechanism* seems to be the same in the two species (cf items 7–11), although there are minor differences whose significance is as yet unknown (cf items 12 and 13) The influence of antirheumatic agents (items 14–18) concern both the *antigen-antibody reaction* and the *biochemical mechanism* (p 41) The experiments with glucocorticoids (items 14 and 15) *show a definite difference between the rat and* the guinea pig while non steroid antirheumatics, *i e* phenylbutazone, sodium salicylate and amidopyrine (*items 16–18*) *indicate no difference* On the basis of these comparisons it can be concluded that a partial accordance exists between the reaction patterns of anaphylactic histamine release in the rat and that in the guinea pig In addition, there is a partial accordance as regards the influence of antirheumatics on anaphylactic histamine release in these two species At the present moment it is not possible to compare with other animal species in particular the man, as very few investigations are available The question of a more general validity of the conclusions obtained in the present study should be evaluated on the basis of similar investigations to be made on other species

The present study may contribute to a better understanding of anaphylactic histamine release and of the species differences This is an essential factor for the comprehension of allergic manifestations, in which histamine release is contributory to the pathogenesis The therapeutic benefits, which such allergic manifestations may gain from especially glucocorticoids, are well established However it still remains to be elucidated whether this effect is due mainly to inhibition of the anaphylactic histamine release

A further consequence of the present study might be the introduction of a new screening test for drugs designed to act on (a) allergic, and (b) rheumatic manifestations As mentioned above, only clinically potent anti rheumatic agents are active in the *in vivo-in vitro* experiments, while an additional effect is obtained with less potent antirheumatics in the *in vitro* experiments. Concerning (a) it should be stressed, that only histamine release is evaluated other factors contributing to the anaphylactic reaction being disregarded Possible species differences should also be seriously considered As regards

(b) it should be emphasized that *non*-antirheumatic agents have not been examined in the experiments described In addition it may be mentioned that at present no evidence points to a mechanism in which anaphylactic hista mine release is involved in the pathogenesis of rheumatic manifestations

Summary

Chapter 1

By far the greater part of the histamine content of the body is bound in the mast cell. During anaphylaxis histamine is released from the mast cell. The released histamine plays a role in eliciting the anaphylactic reaction.

Antirheumatic agents can protect certain animals from fatal anaphylactic shock. Thus cortisone protects the rat, but not the guinea pig; salicylates, acetylsalicylic acid, and amidopyrine protect the rabbit.

The role of histamine in anaphylactic shock and the ability of antirheumatic agents to counteract this shock motivated the present author's studies performed to investigate whether these agents are able to inhibit the anaphylactic histamine release. The problem posed, then, was to elucidate the mechanism of the anaphylactic histamine release, *int al* and in particular by investigating the effect of antirheumatics upon the histamine release. Another aim was to work out a method for investigating the histamine release in anaphylactic reaction *in vitro*. The author points out the limitation due to the fact that his experiments were done exclusively on rats.

Chapter 2

An account is given of the main phases in anaphylactic histamine release, viz (a) antibody formation (the formation of mast cell sensitizing antibody), (b) the antigen antibody reaction, and (c) the biochemical mechanism elicited in the mast cell by the antigen antibody reaction and resulting in release of mast-cell granules, and thereby release of the histamine content of the cell.

Other authors have previously shown that in the rat, the method of sensitization is all important in respect to the degree of anaphylactic hypersensitivity obtained. Thus, a marked hypersensitivity, resulting in fatal anaphylactic shock and pronounced histamine release caused by challenge with specific antigen, can be obtained only when the sensitization with antigen is accompanied by a simultaneous administration of *Bordetella pertussis* bacteria.

Anaphylactic histamine release *in vitro* has been based upon experiments using suspended tissue, perfused tissue, or suspensions of peritoneal cells. The last mentioned method was used by the present author, because it affords several advantages, *int al* a possibility of determining the mast cell

(b) it should be emphasized that *non* antirheumatic agents have not been examined in the experiments described In addition it may be mentioned that at present no evidence points to a mechanism in which anaphylactic hista mine release is involved in the pathogenesis of rheumatic manifestations

mine liberators does release histamine by triggering-off the same biochemical mechanism as the antigen antibody *reaction*

Chapter 4

The present author has studied the influence of antirheumatic agents upon the anaphylactic histamine release in the rat by *in vivo-in vitro* experiments and by *in vitro* experiments In both sorts of experiments steroid as well as non steroid antirheumatics were able to inhibit histamine release In the *in vivo-in vitro* experiments only clinically potent antirheumatics, *i e* glucocorticoids, sodium *aurothiosulphate and* phenylbutazone acted In the *in vitro* experiments an effect was also obtained with weaker antirheumatics, *i e* sodium salicylate, sodium γ-resorcylate and amidopyrine, in high concentrations *Such high concentrations cannot be obtained in vivo* with reasonable doses In both instances the order of potency was the same (sodium aurothiosulphate > hydrocortisone > phenylbutazone > salicylate and amidopyrine) excep [illegible] aurothiosulphate which *did not* act *in vitro*

In the *in vi* [illegible]

mechanisms, [illegible]

antirheumatic agent was administered Thus, *hydrocortisone inhibited the* anaphylactic histamine release when given at the beginning or towards the end of the sensitization period During the initial phase the mechanism de*pended on inhibition of the formation of mast cell-sensitizing antibody* During the late phase it was due to inhibition of the antigen-antibody reaction or inhibition of the biochemical mechanism, or of both *Phenylbutazone* and *sodium aurothiosulphate* inhibited the anaphylactic *histamine* release *only* when given towards the end of the sensitization period The mechanism is an inhibition of the antigen antibody reaction, of the biochemical mechanism or of both Administration of *hydrocortisone, phenylbutazone and* sodium aurothiosulphate prior to the sensitization period does not influence the histamine release

In the *in vitro* experiments hydrocortisone, *phenylbutazone, sodium* salicylate, sodium γ-resorcylate and amidopyrine inhibited the anaphylactic histamine release by a mechanism depending on inhibition of the antigen-antibody reaction, of the biochemical mechanism, or of both

By means of *in vivo in vitro* experiments it is possible to distinguish between *the mechanisms (a)*, an inhibition of the antibody formation, and (b), an inhibition of the antigen antibody reaction, or/and *an inhibition of* the biochemical mechanism In contrast to this, the *in vitro* experiments can only reveal a *mechanism depending* on (b) An attempt to distinguish between the antigen antibody reaction and the biochemical *mechanism* by means of histamine liberators showed that this was not possible

concentration Various methods for histamine assay are mentioned In the present study two chemical methods were adapted for determination in peritoneal cell suspensions from rats, since we found these methods specific and sufficiently sensitive for this purpose The author has then standardized a method for measuring histamine release caused by antigen in the mentioned cell suspensions from sensitized rats That the method is well suited for evaluation of anaphylactic histamine liberation was thereafter established by examinations which resulted in the following facts the content of various cells in the peritoneal cell suspension is demonstrated, and it is established that the histamine content is derived almost exclusively from the mast cells. The release of histamine caused by antigen is due to a specific antigen-antibody reaction, as non specific antigens do not release histamine The anaphylactic histamine release is high, and the spontaneous histamine release slight The author could not confirm the possibility that other cells might release a factor during anaphylaxis, a factor which then releases histamine from the mast cell. There is thus every probability that the histamine release by the mast cell is not affected by other cells or substances in the peritoneal cell suspension Accordingly, antigen releases histamine by reacting only with the mast cell, thus, isolation of mast cells, which might lead to a change in their sensitivity to antigen, is avoided The concentration of mast cells in the suspension is of no significance to the anaphylactic histamine release, in other words, the same percentage of the total histamine content of the sample will be released at different concentrations of mast cells

For anaphylactic histamine release we found a relationship between the antigen concentration and the histamine release Thus, the anaphylactic histamine release increases in proportion to the logarithm of the antigen concentration Furthermore it is established that anaphylactic histamine release is a rapid process

Chapter 3

The hypothesis has been advanced by UVNÄS and his colleagues that certain histamine liberators – such as compound 48/80, lecithinase A, and α chymotrypsin – liberate histamine from the rat mast cells by triggering-off the same biochemical mechanism in the mast cell as the antigen-antibody reaction The present study shows that this hypothesis is not quite correct Thus, in cases where rats have been pre-treated by the same antirheumatic substance, the histamine release caused by the named histamine liberators is nevertheless influenced in different ways, which shows that the histamine liberators must release histamine by eliciting different biochemical mechanisms From the present experiments it is not possible to deduce whether one of these hista-

REFERENCES

Aaborg C. H, J Novotny & B Uvnas Ionic binding of histamine in mast cell granules *Acta physiol scand* 1967, 69, 276–283

Alcasu, A. & G B West Anaphylaxis in the dog *Int Arch Allergy* 1960, 16, 326–335

Arbesman, C E, E Neter & L. F Bertram The effect of ACTH and cortisone on certain immunologic mechanisms including reversed anaphylaxis *J Allergy* 1951, 22 340–349

Austen K F Immunological aspects of salicylate action – a review In *salicylates an international symposium*, ed. Dixon et al, Churchill, London 1963, p 161–169

Barger, G & H H. Dale β-Iminazolylethylamine a depressor constituent of intestinal mucosa. *J Physiol* 1911, 41, 499–503

Barsoum, G S & J H Gaddum The pharmacological estimation of adenosine and histamine in blood *J Physiol* 1935, 85, 1–14

Bartosch R., W Feldberg & E. Nagel Das Freiwerden eines histaminahnlichen Stoffes bei der Anaphylaxis des Meerschweinchens *Pflugers Archiv f d ges Physiol* 1932, 230, 129–153

Becker E L Nature and significance of antigen antibody activated esterases In *Biochemistry of the acute allergic reactions*, Blackwell Scientific publications, Oxford, ed. Austen, K. F & E. L. Becker 1968, p 199–213

Becker E L & K F Austen A comparison of the specificity of inhibition by phosphonate esters of the first component of complement and the antigen – induced release of histamine from guinea pig lung *J Exp Med* 1964, 120, 491–506

Becker E L & K F Austen Mechanisms of immunologic injury of rat peritoneal mast cells I The effect of phosphonate inhibitors on the homocytotropic antibody – mediated histamine release and the first component of rat complement. *J Exp Med* 1966, 124 379–395

Benacerraf B Properties of immunoglobulins which mediate the release of vasoactive amines in experimental animals In *Immunopharmacology*, ed H O Schild, Pergamon Press, Oxford 1968 p 3–13

Benacerraf B, Z Ovary, K J Bloch & E. C. Franklin Properties of guinea pig 7 S antibodies I Electrophoretic separation of two types of guinea pig 7 S antibodies *J Exp Med* 1963, 117, 937–949

Bertola, G & L. Castellani Influenza del fenilbutazone e del cortisone sullo shock anafilattico da siero nella cavia. *Arch E Maragliano Pat clin.* 1958, 14, 107–113

Binaghi R. A., B Benacerraf, K. J Bloch & F M Kourilsky Properties of rat anaphylactic antibody *J Immunol* 1964, 92, 927–933

Bray, R. E. & P P van Arsdel Jr In vitro histamine release from rat mast cells by chemical and physical agents. *Proc Soc Exp Biol Med* 1961, 106, 255–259

Chapter 5

In the present study a method was developed for the purpose of evaluating anaphylactic histamine release in the rat *in vitro* It was established that the *method satisfied the requirements made on such an evaluation*

The author has shown that certain antirheumatic substances – steroids as well as non-steroids – inhibit anaphylactic histamine release The mechanism of action for this inhibition was examined, and it was found to be due to different mechanisms, depending on the time of the sensitization period at which the antirheumatic agent was administered

The species difference and the general validity of the conclusions are discussed

REFERENCES

Aaborg C. H J Novotny & B Uvnas Ionic binding of histamine in mast cell granules *Acta physiol scand* 1967, 69, 276–283

Akcasu A. & G B West Anaphylaxis in the dog. *Int Arch Allergy* 1960, 16, 326–335

Arbesman C E E Neter & L. F Bertram The effect of ACTH and cortisone on certain immunologic mechanisms including reversed anaphylaxis. *J Allergy* 1951, 22 340–349

Austen K F Immunological aspects of salicylate action – a review In *salicylates an international symposium* ed Dixon et al Churchill, London 1963, p 161–169

Barger G & H H Dale β-Iminazolylethylamine a depressor constituent of intestinal mucosa *J Physiol* 1911, 41 499–503

Barsoum G S & J H. Gaddum The pharmacological estimation of adenosine and histamine in blood *J Physiol* 1935 85, 1–14

Bartosch R W Feldberg & E Nagel Das Freiwerden eines histaminahnlichen Stoffes bei der Anaphylaxis des Meerschweinchens *Pflugers Archiv f d ges Physiol* 1932 230 129–153

Becker E L Nature and significance of antigen antibody activated esterases In *Biochemistry of the acute allergic reactions* Blackwell Scientific publications, Oxford ed. Austen K F & E L Becker 1968, p 199–213

Becker E L & K F Austen A comparison of the specificity of inhibition by phosphonate esters of the first component of complement and the antigen – induced release of histamine from guinea pig lung *J Exp Med* 1964, 120, 491 506

Becker E L & K F Austen Mechanisms of immunologic injury of rat peritoneal mast cells I The effect of phosphonate inhibitors on the homocytotropic antibody – mediated histamine release and the first component of rat complement *J Exp Med* 1966 124 379–395

Benacerraf B Properties of immunoglobulins which mediate the release of vasoactive amines in experimental animals. In *Immunopharmacology* ed H O Schild, Pergamon Press, Oxford 1968 p 3–13

Benacerraf B Z Ovary K. J Bloch & E. C. Franklin Properties of guinea pig 7 S antibodies I Electrophoretic separation of two types of guinea pig 7 S antibodies *J Exp Med* 1963 117, 937–949

Bertola G & L Castellani Influenza del fenilbutazone e del cortisone sullo shock anafilattico da siero nella cavia *Arch E Maragliano Pat clin.* 1958, 14, 107–113

Binaghi R A. B Benacerraf, K. J Bloch & F M Kourilsky Properties of rat anaphylactic antibody *J Immunol* 1964, 92, 927–933

Bray R E. & P P van Arsdel Jr In vitro histamine release from rat mast cells by chemical and physical agents. *Proc Soc Exp Biol Med* 1961, 106, 255–259

Brocklehurst, W E The release of histamine and formation of a slow reacting substance (SRS A) during anaphylactic shock *J Physiol* 1960, **151**, 416–435

Brocklehurst, W E & S C Lahiri The production of bradykinin in anaphylaxis. *J Physiol* 1962, **160**, 15P–16P

Brocklehurst, W E & S C Lahiri Formation and destruction of bradykinin during anaphylaxis *J Physiol* 1963, **165**, 39P–40P

Campbell, B Inhibition of anaphylactic shock by acetylsalicylic acid *Science* 1948 **108**, 478–479

Carlini, E A & J P Green The subcellular distribution of histamine, slow reacting substance and 5 hydroxytryptamine in the brain of the rat *Brit J Pharmacol* 1963, **20**, 264–277

Chakravarty, N The mechanism of histamine release in anaphylactic reaction in guinea pig and rat *Acta physiol scand* 1960, **48**, 146–166

Chakravarty, N Inhibition of anaphylactic histamine release by 2 deoxyglucose *Nature* 1962, **194**, 1182–1184

Chakravarty, N Respiration of rat peritoneal mast cells during histamine release induced by antigen antibody reaction *Exptl Cell Res* 1968, **49**, 160–168

Chakravarty, N Further observations on the inhibition of histamine release by 2 deoxyglucose *Acta physiol scand* 1968b, **72**, 425–432

Chakravarty, N & B Uvnas Histamine and a lipid soluble smoothmuscle stimulating principle (SRS) in anaphylactic reaction *Acta physiol scand* 1960, **48**, 302–14

Code C F The quantitative estimation of histamine in the blood *J Physiol* 1937 **89**, 257–268

Code, C F, D T Cody, M Hurn, J C Kennedy & M J Strickland The simultaneous release of histamine and a histamine – destroying factor during anaphylaxis in rats *J Physiol* 1961, **156**, 207–216

Code, D T, C F Code & J C. Kennedy Studies on the mechanism of anaphylaxis in the rat *J Allergy* 1963, **34**, 26–34

Collier, H O J Release of histamine – clinical considerations In *Histamine, Ciba Foundation Symposium* ed Wolstenholme, G E W & C M O Connor Churchill Ltd, London 1956, p 155–159

Collier, H O J & G W L. James Humoral factors affecting pulmonary inflation during acute anaphylaxis in the guinea pig in vivo *Br J Pharmac Chemother* 1967, **30**, 283–301

Dale H H The anaphylactic reaction of plain muscle in the guinea pig *J Pharmacol Exp Therap* 1913, **4**, 167–223

Dale, H H & P P Laidlaw The physiological action of β iminazolylethylamine *J Physiol* 1910, **41**, 318–344

Diamant, B Observations on anaphylactic and compound 48/80 induced histamine release from guinea pig and rat lung tissue in vitro *Acta physiol scand* 1962a **55** 11–25

Diamant, B Betydelsen av glykos vid histaminfrisättning och mastcells degranulering En experimentell studie på isolerad lungvavnad och mastceller *Thesis* Karolinska Institutet, Stockholm 1962b

Diamant, B & P G Kruger Histamine release from isolated rat peritoneal mast cells induced by adenosine 5 triphosphate *Acta physiol scand* 1967, **71** 291–302

Diamant, B The influence of adenosine triphosphate on isolated rat peritoneal mast cells *Int Arch Allergy* 1969, **36**, 3–21

Dragstedt, C A & E. Gebauer Fuelnegg Studies in anaphylaxis I The appearance of a physiologically active substance during anaphylactic shock. *Amer J Physiol* 1932, 102, 512–19

Dworetzky, M, C F Code & G H Higgins Effect of cortisone and ACTH on eosinophils and anaphylactic shock in guinea pigs. *Proc. Soc Exp Biol Med* 1950, 75, 201–206

Feldberg W & C. H Kellaway Liberation of histamine from the perfused lung by snake venoms *J Physiol* 1937, 90, 257–279

Feldberg W & J Talesnik Reduction of tissue histamine by compound 48 80 *J Physiol.* 1953 120, 550–568.

Garcia Arocha, H. Liberation of 5 hydroxytryptamine and histamine in the anaphylactic reaction of the rat. *Can J Biochem Physiol.* 1961, 39, 403–416

Gebauer Fuelnegg E. & C. A. Dragstedt. Studies in anaphylaxis II The nature of a physiologically active substance appearing during anaphylactic shock. *Amer J Physiol* 1932 102 520–26

Germuth F G Jr The role of adrenocortical steroids in infection, immunity and hypersensitivity *Pharmacol. Rev* 1956, 8, 1–24

Germuth, F G Jr & B Ottinger Effect of 17 Hydroxy 11-dehydrocorticosterone (Compound E) and of ACTH on Arthus reaction and antibody formation in the rabbit. *Proc soc Exper Biol & Med* 1950 74, 815–823

Germuth F G Jr B Ottinger & J Oyama Influence of cortisone on experimental hypersensitivity and circulating antibody in the guinea pig *Proc Soc Exper Biol & Med* 1952, 80 188–191

Goadby P & W G Smith Observations on the anti anaphylactic activity of hydrocortisone and related steroids *J Pharm Pharmacol* 1964, 16, 108–114

Goth A Effect of drugs on mast cells. *Advances in Pharmacology* 1967, 5 47–78

Graham, H T O H Lowry, F Wheelwright, M A. Lenz & H H. Parish, Jr Distribution of histamine among leukocytes and platelets. *Blood* 1955, 10, 467–481

Greaves M W & J L. Mongar Mechanism of the anaphylactic reaction. In *Immunopharmacology* ed H. O Schild, Pergamon Press, Oxford 1968, p 45–63

Green, A F The antagonism of histamine and the anaphylactic response by phenyl pyridyl allylamines. *Brit J Pharmacol* 1953, 8, 171–176

Halpern B N Liacopoulos P & C P del Castillo L'anaphylaxie expérimentale chez le Rat albinos. *Compt rend soc biol* 1955, 149, 314–319

Humphrey J H & R. Jaques The histamine and serotonin content of the platelets and polymorphonuclear leucocytes of various species. *J Physiol* 1954, 124, 305–10

Humphrey J H & I Mota The mechanism of anaphylaxis *Specificity of antigen – induced mast cell damage in anaphylaxis in the guinea pig. Immunology* 1959, 2, 31–43

Hogberg, B & B Uvnas Inhibitory action of allicin on degranulation of mast cells produced by compound 48/80 histamine liberator from ascaris, lecithinase A, and antigen *Acta physiol scand.* 1958, 44 157–162.

Hogberg, B & B Uvnas Further observations on the disruption of rat mesentery mast cells caused by compound 48/80, antigen antibody reaction, lecithinase A and decylamine. *Acta physiol scand* 1960, 48 133–145.

Johnson, A. R. & N C. Moran Comparison of several methods for isolation of rat peritoneal mast cells. *Proc Soc. Exp Biol Med* 1966, 123 886–889

Kahl, G F & K J Netter Sauerstoffverbrauch isolierter peritonealmastzellen der ratte wahrend der histaminfreisetzung durch compound 48/80 *Naun Arch Pharm Exptl Path* 1967, **256**, 55–63

Keller, R & I Beeger Das Verhalten isolierter sensibilisierter Mastzellen nach Einwirkung des Antigens unter verschieden Versuchsbedingungen *Med exp* 1961, 4 51–58

Kremzner, L. T & C C Pfeiffer Identification of substances interfering with the fluorometric determination of brain histamine *Biochem Pharmacol* 1966 **15** 197–200

Lagunoff, D & E P Benditt 5 Hydroxytryptophan decarboxylase activity in rat mast cells *Amer J Physiol* 1959, **196**, 993–997

Lagunoff, D & E P Benditt Mast cell degranulation and histamine release observed in a new in vitro system *J Exp Med* 1960, **112**, 571–579

Lepper, M H, E R Caldwell, Jr, P K Smith & B F Miller Effects on anaphylactic shock of salicylates, aminopyrine and other chemically and pharmacologically related compounds *Proc Soc exp Biol* 1950, **74**, 254–258

Lipton, M M, S H Stone & J Freund Systemic and local anaphylaxis in the albino rat *J Immunol* 1956, **77**, 453–461

Lowry, O H, H T Graham, F B Harris, M K Priebat, A R Marks & R U Bregman The chemical measurement of histamine in blood plasma and cells *J Pharmacol* 1954, **112**, 116–126

Macintosh, F C & W D M Paton The liberation of histamine by certain organic bases *J Physiol* 1949, **109**, 190–219

Malkiel, S & B J Hargis Histamine sensitivity and anaphylaxis in the pertussis – vaccinated rat *Proc Soc Exper Biol & Med* 1952, **81**, 689–691

Mongar, J L & H O Schild Inhibition of the anaphylactic reaction *J Physiol* (London) 1957, **135**, 301–319

Mongar, J L & H O Schild The effect of calcium and pH on the anaphylactic reaction *J Physiol* 1958, **140**, 272–284

Mongar, J L & B A V Perera Oxygen consumption during histamine release by antigen and compound 48/80 *Immunology* 1965, **8**, 511–518

Mongar, J L & H O Schild Cellular mechanisms in anaphylaxis *Physiol Rev* 1962, **42**, 226–270

Mota, I Action of anaphylactic shock and anaphylatoxin on mast cells and histamine in rats *Brit J Pharmacol* 1957, **12**, 453–456

Mota I Mast cell and histamine in rat anaphylaxis The effect of Haemophilus pertussis *Nature* 1958, **182**, 1021–1022

Mota, I Mast cell lytic antibodies *Nature* 1961, **192**, 1201

Mota, I Failure of rat and rabbit antiserum to passively sensitize normal and pertussis – treated rats and mice so as to induce mast cell damage and histamine release on later contact with antigen *Immunology* 1962 **5**, 11–19

Mota, I Mast cells and anaphylaxis *Ann New York Acad Sciences* 1963, **103**, 264–76

Mota I The behaviour of mast cells in anaphylaxis *Int Rev Cytol* 1963 b **15** 363–397

Mota, I Biological characterization of mast cell sensitizing' antibodies *Life Sciences* 1963 c 1 465–74

Mota, I The mechanism af anaphyl axis I Production and biological properties of "mast cell sensitizing" antibody *Immunology* 1964, **7**, 681–699

Mota, I The mechanism of anaphylaxis. II. The role of "mast cell sensitizing" antibody and delayed hypersensitivity in rat anaphylaxis. *Immunology* 1964b, 7 700–706

Mota I Properties of rat and mouse homocytotropic antibodies. In *Biochemistry of the acute allergic reactions* p 189–197, 1968 Blackwell Scientific Publications. Ed K. F Austen & E. L. Becker

Mota, I & Tisue Ishu Inhibition of mast cell disruption and histamine release in rat anaphylaxis in vitro Comparison with compound 48/80 *Brit J Pharmacol* 1960 15 82–87

Mota I & W Dias da Silva Antigen – induced damage to isolated sensitized mast cells. *Nature* 1960 186, 245–246

Naranjo P Toxicity of histamine lethal doses In *Handbook of experimental pharmacology* (vol 18/1), ed Eichler, O & A. Farah, Springer Verlag, Berlin 1966 p 179–201

Nelson C. T., C. L. Fox, Jr & E. B Freeman. Inhibitory effect of cortisone on anaphylaxis in the mouse. *Proc Soc Exper Biol & Med* 1950, 75, 181 183

Norn S Influence of antirheumatic agents on the release of histamine from rat peritoneal mast cells after an antigen antibody reaction *Acta pharmacol et toxicol* 1965 a 22 369–378

Norn S Chemical determination of histamine in tissues and mast cells *Acta pharmacol et toxicol* 1965 b 22 115–125

Norn S Release of histamine from sensitized rat peritoneal cells by specific and unspecific antigens. *Acta pharmacol et toxicol* 1967 a, 25 456–460

Norn S Effect of histamine liberators on rat peritoneal mast cells and influence of antirheumatic agents. *Acta pharmacol et toxicol* 1967 b, 25, 281–289

Norn S Antigenic histamine release from fractionated and unfractionated peritoneal cells from sensitized rats. *Acta pharmacol et toxicol* 1968 a 26, 373–383

Norn S Influence of antirheumatic agents on the release of histamine from sensitized rat peritoneal mast cells II Antibody production. *Acta pharmacol et toxicol* 1968 b 26 470–474

Norn, S Influence of antirheumatic agents on histamine release from sensitized rat peritoneal mast cells. Mechanism of action. In *Inflammation Biochemistry and Drug Interaction* ed. A Bertelli & J C Houck. Excerpta Medica International Congress Series No 188 1969 pp 218–220 International Symposium Como Italy Oct. 1968

Ovary Z B Benacerraf & K J Bloch Properties of guinea pig 7 S antibodies II Identification of antibodies involved in passive cutaneous and systemic anaphylaxis. *J Exp Med* 1963 117 951–964

Parratt, J R. & G B West Release of 5 hydroxy tryptamine and histamine from tissues of the rat. *J Physiol* 1957 137, 179–192.

Paton W D M Compound 48/80 A potent histamine liberator *Brit J Pharmacol* 1951 6 499–508.

Paton W D M Histamine release by compounds of simple chemical structure. *Pharmacol Rev* 1957 9 269–328

Perera, B A V & J L Mongar The role of a chymotrypsin – like enzyme in histamine release from rat mast cells. *Immunology* 1963 6 472–477

Perera, B A V & J L. Mongar The role in anaphylaxis of a chymotrypsin – like enzyme in rat mast cells. *Immunology* 1963 b, 6, 478–483

Persellin, R. H., E. V Hess & M Ziff Effect of a gold salt on the immune response *Arthritis and Rheum.* 1967, 10, 99–106

Kahl, G F & K J Netter· Sauerstoffverbrauch isolierter peritonealmastzellen der ratte wahrend der histaminfreisetzung durch compound 48/80 *Naun Arch Pharm Exptl Path* 1967, **256**, 55–63

Keller, R & I Beeger Das Verhalten isolierter sensibilisierter Mastzellen nach Ein wirkung des Antigens unter verschieden Versuchsbedingungen *Med exp* 1961, **4** 51–58

Kremzner, L. T & C C Pfeiffer Identification of substances interfering with the fluorometric determination of brain histamine *Biochem Pharmacol* 1966, **15** 197–200

Lagunoff, D & E P Benditt 5 Hydroxytryptophan decarboxylase activity in rat mast cells *Amer J Physiol* 1959, **196**, 993–997

Lagunoff, D & E P Benditt Mast cell degranulation and histamine release observed in a new in vitro system *J Exp Med* 1960, **112**, 571–579

Lepper, M H, E R Caldwell, Jr, P K Smith & B F Miller Effects on anaphylactic shock of salicylates, aminopyrine and other chemically and pharmacologically related compounds *Proc Soc exp Biol* 1950, **74**, 254–258

Lipton M M, S. H Stone & J Freund Systemic and local anaphylaxis in the albino rat *J Immunol* 1956, **77**, 453–461

Lowry, O H, H T Graham, F B Harris, M K. Priebat, A R Marks & R U Bregman The chemical measurement of histamine in blood plasma and cells *J Pharmacol* 1954, **112**, 116–126.

Macintosh, F C & W D M Paton The liberation of histamine by certain organic bases *J Physiol* 1949, **109**, 190–219

Malkiel, S & B J Hargis Histamine sensitivity and anaphylaxis in the pertussis – vaccinated rat *Proc Soc Exper Biol & Med* 1952, **81**, 689–691

Mongar, J L & H O Schild Inhibition of the anaphylactic reaction *J Physiol* (London) 1957, **135**, 301–319

Mongar, J L. & H O Schild The effect of calcium and pH on the anaphylactic reaction *J Physiol* 1958, **140**, 272–284

Mongar, J L & B A V Perera Oxygen consumption during histamine release by antigen and compound 48/80 *Immunology* 1965, **8** 511–518

Mongar, J L & H O Schild Cellular mechanisms in anaphylaxis *Physiol Rev* 1962, **42**, 226–270

Mota I Action of anaphylactic shock and anaphylatoxin on mast cells and histamine in rats *Brit J Pharmacol* 1957, **12**, 453–456

Mota I Mast cell and histamine in rat anaphylaxis The effect of Haemophilus pertussis *Nature* 1958, **182**, 1021–1022.

Mota, I Mast cell lytic antibodies *Nature* 1961, **192**, 1201

Mota, I Failure of rat and rabbit antiserum to passively sensitize normal and pertussis – treated rats and mice so as to induce mast cell damage and histamine release on later contact with antigen *Immunology* 1962, **5**, 11–19

Mota I Mast cells and anaphylaxis *Ann New York Acad Sciences* 1963 **103** 264–76

Mota, I The behaviour of mast cells in anaphylaxis *Int Rev Cytol* 1963 b **15** 363–397

Mota, I Biological characterization of mast cell sensitizing" antibodies *Life Sciences* 1963 c **1** 465–74

Mota, I The mechanism af anaphyl axis I Production and biological properties of "mast cell sensitizing" antibody *Immunology* 1964, **7**, 681–699

Uvnas, B & I L. Thon Isolation of "biologically intact" mast cells *Exptl Cell Research* 1959, 18, 512–20

Uvnas, B & I L. Thon A physico chemical model of histamine release from mast cells. In *Mechanisms of release of biogenic amines* ed U S Von Euler; S Rosell & B Uvnas, Pergamon Press, London 1966, p 361 369

Valentine W N., J S. Lawrence, M Lee Pearce & W S Beck. The relationship of the basophil to blood histamine in man. *Blood* 1955, 10, 154–159

Waksman, B H Specific white cell lysis produced by combination of rabbit anti serum to purified protein (ovalbumin, bovine gamma globulin) with homologous antigen The role of non precipitating antibody *J Immunology* 1953, 70 331–344

Windaus A & W Vogt Synthese des Imidazolylathyl amins *Ber Dtsch Chem Ges* 1907, 40, 3691–3695

Raffel, S Immediate hypersensitivities Immunity Appleton – Century – Crofts, Inc New York 1961, p 198–244

Rechenberg H K Phenylbutazone Edward Arnold (Publishers), London 1962

Riley, J F & G B West The presence of histamine in tissue mast cells *J Physiol* 1953, 120, 528–37

Rother, K & F Katz Experimentelle untersuchungen uber die beeinflussung der anti korperbildung durch butazolidin *Arch int pharmacodyn* 1957, 112, 65–72

Sanyal, R K & G B West The relationship of histamine and 5 hydroxytryptamine to anaphylactic shock in different species *J Physiol* 1958, 144, 525–31

Schild, H O Histamine release in anaphylactic shock from various tissues of the guinea pig *J Physiol*, London 1939, 95, 393–403

Schmutzler, W, H Giertz & F Hahn Neue Untersuchungen uber die Rolle des Histamins bei der Meerschweinchenanaphylaxie *Int Arch Allergy* 1963, 22 69–76

Seegers, W & Aa Janoff Mediators of inflammation in leukocyte lysosomes VI Partial purification and characterization of a mast cellrupturing component *J exp Med* 1966, 124, 833–849

Shore, P A, A. Burkhalter & V H Cohn, Jr A method for the fluorometric assay of histamine in tissues *J Pharmacol* 1959, 127, 182–186

Smith, W & J H Humphrey The effect of sodium salicylate upon hypersensitivity reactions *Brit J exp Path* 1949, 30, 560–571

Smull, K, R. W Wissler & J M Watson The effect of sodium salicylate upon serum disease in rabbits *J Lab clin Med* 1948, 33, 936–950

Stechschulte, D J, K F Austen & K J Bloch Antibodies involved in antigen – induced release of slow reacting substance of anaphylaxis (SRS A) in the guinea pig and rat *J Exp Med* 1967, 125, 127–147

Swift, H F The action of sodium salicylate upon the formation of immune bodies *J exp Med* 1922, 36, 735–760

Tabor, H & C. W Tabor· Spermidine, spermine, and related amines *Pharmacol Rev* 1964, 16, 245–300

Telford, J M & B West Some aspects of experimental allergy part I *Int Arch Allergy* 1963, 22, 11–30

Tigano, F Ricerche sperimentali intorno all attivita del fenilbutazone su alcuni fenomeni allergici nota I Fenilbutazone e shock anafilattico *G Batt Immun* 1955 48 134–148

Trethewie, E R The influence of sodium salicylate and acetyl salicylic acid on the release of histamine in anaphylaxis. *Aust J exp biol med Sci* 1951, 29 443–450

Trethewie, E R The effect of butazolidine on the anaphylactic lung *Austral J exp Biol* 1957, 35, 541–547

Trethewie, E R. Cortisone and anaphylaxis *Austral J exp Biol* 1958 36 275–283

Udenfriend, S & T P Waalkes On the role of serotonin in anaphylaxis In *International Symposium on Mechanisms of Hypersensitivity* ed J H Shaffer, G A Lo Grippo & M W Chase Churchill, London 1959 p 219–226

Uvnas, B Mechanism of histamine release *Proc Int Union Physiol Sci* XXII Int Congr Leiden 1962, p 106–114

Uvnas, B Mechanism of histamine release in mast cells *Ann N Y Acad Sci* 1963 103, 278–284

Uvnas, B Der Mechanismus der Histaminfreisetzung aus Mastzellen Naunyn Schmiedebergs *Arch exp Path Pharmak* 1965, 250, 137–149

Uvnas, B & J Antonsson Triggering action of phosphatidase A and chymotrypsins on degranulation of rat mesentery mast cells *Biochem Pharmacol* 1963 12 867–873

Uvnas, B & I L. Thon Isolation of "biologically intact" mast cells *Exptl Cell Research* 1959, 18, 512–20

Uvnas, B & I L. Thon A physico chemical model of histamine release from mast cells In *Mechanisms of release of biogenic amines*, ed. U S Von Euler; S Rosell & B Uvnas, Pergamon Press, London 1966, p 361–369

Valentine, W N., J S Lawrence, M Lee Pearce & W S. Beck. The relationship of the basophil to blood histamine in man. *Blood* 1955, 10, 154–159

Waksman, B H Specific white cell lysis produced by combination of rabbit anti-serum to purified protein (ovalbumin, bovine gamma globulin) with homologous antigen The role of non precipitating antibody *J Immunology* 1953, 70, 331–344

Windaus, A & W Vogt Synthese des Imidazolyläthyl amins *Ber Dtsch Chem Ges* 1907 40, 3691–3695

Dansk resumé

Kapitel 1

Langt hovedparten af organismens histaminindhold er bundet i *mastcellen*. Under anafylaksi frigøres histamin fra mastcellen. Det frigjorte histamin er af betydning for udløsning af den anafylaktiske *reaktion*.

Antirheumatiske substanser kan beskytte visse dyr mod fatalt anafylaktisk shock. Kortison beskytter således rotten, men ikke marsvinet, og salicylater, acetylsalicylsyre og amidopyrin beskytter kaninen.

Histaminets betydning for det anafylaktiske *shock* og antirheumatiske *stoffers* evne *til* at modvirke dette shock motiverer forfatterens undersøgelse af, om disse farmaka kan hæmme den anafylaktiske histaminfrigørelse. Problemstillingen består derfor i at belyse mekanismen ved den anafylaktiske histaminfrigørelse, bl.a. og især ved at undersøge indvirkningen af antirheumatika på histaminfrigørelsen. Hertil kommer at udarbejde *en metode til undersøgelse af histaminfrigørelsen* ved den anafylaktiske reaktion *in vitro*. Forfatteren gør opmærksom på den begrænsning, der ligger i, at hans forsøg udelukkende er baseret på rotteeksperimenter.

Kapitel 2

Der gives en oversigt over hovedfaserne i den anafylaktiske histaminfrigørelse. Disse omfatter (a) antistofdannelse (dannelse af mastcelle sensibiliserende antistof), (b) antigen-antistofreaktionen og (c) den biokemiske mekanisme, som udløses i mastcellen ved antigen-antistofreaktionen, og som resulterer i en frigørelse af mastcellens granula og hermed frigørelse af cellens histaminindhold.

Andre forfattere har tidligere vist, at hos rotten er sensibiliseringsmetoden helt afgørende for den opnåelige grad af anafylaktisk hypersensibilitet. Således kan der kun opnås en kraftig hypersensibilitet resulterende i fatalt anafylaktisk shock og kraftig histaminfrigørelse ved provokation med antigen, når sensibiliseringen med antigen ledsages af indgift af Bordetella pertussis bakterier.

Anafylaktisk histaminfrigørelse *in vitro* har været baseret på eksperimenter med suspenderet væv, perfunderet væv eller på suspensioner af peritoneal celler. Sidstnævnte metode er anvendt af forfatteren, fordi den byder på flere fordele, bl.a. muligheden for bestemmelse af mastcellekoncentrationen

Forskellige metoder til bestemmelse af histamin omtales I nærværende arbejde har der været anvendt to kemiske metoder til måling i peritonealcellesuspensioner fra rotter Bestemmelserne er fundet specifikke og tilstrækkeligt følsomme til dette formål Forfatteren har herefter standardiseret en metode til måling af histaminfrigørelse, forårsaget af antigen i nævnte cellesuspension fra sensibiliserede rotter Det er herefter blevet godtgjort, at metoden er velegnet til bedømmelse af anafylaktisk histaminfrigørelse ved hjælp af undersøgelser, som viste følgende forhold indholdet af forskellige celler i peritoneal cellesuspensionen er klarlagt, og det fastslås, at histaminindholdet stammer næsten udelukkende fra mastcellerne Histaminfrigørelsen, forårsaget af antigen, skyldes en specifik antigen-antistofreaktion, idet uspecifikke antigener ikke frigør histamin Den anafylaktiske histaminfrigørelse er stor, og den spontane histaminfrigørelse er ringe Den mulighed, at andre celler skulle frigøre en faktor under anafylaksi, som så frigør histamin fra mastcellen kunne ikke bekræftes Det er derfor overvejende sandsynligt, at mastcellens histaminfrigørelse ikke påvirkes af de øvrige celler eller substanser i peritonealcellesuspensionen Antigenet må derfor frigøre histamin ved at reagere udelukkende med mastcellen En isolering af mastcellen er derfor overflødig. Dette er væsentligt, fordi forskellige isoleringsprocedurer kan resultere i en ændring af mastcellens følsomhed overfor antigen Koncentrationen af mastceller i suspensionen er uden betydning for den anafylaktiske histaminfrigørelse, d v s den samme procentdel af prøvens totale histaminindhold frigøres ved forskellig koncentration af mastcellen

Forfatteren har fundet en sammenhæng mellem antigenkoncentrationen og den anafylaktiske histaminfrigørelse Således stiger den anafylaktiske histaminfrigørelse proportionalt med logaritmen til antigenkoncentrationen Yderligere er det vist at den anafylaktiske histaminfrigørelse er en hurtig proces

Kapitel 3

Den hypotese har været fremsat af UVNÄS og medarbejdere, at visse histaminliberatorer som compound 48/80, lecithinase A og α-chymotrypsin skulle frigøre histamin fra rottens mastceller *ved at udløse den samme biokemiske mekanisme* i mastcellen som antigen-antistofreaktionen Nærværende *arbejde viser* at denne hypotese ikke er korrekt Således influeres *histaminfrigørelsen* ved de nævnte histaminliberatorer på *forskellig måde, når rotten forbehandles* med det samme antirheumatiske stof, *hvilket viser, at histaminliberatorerne* må frigøre histamin ved at udløse *forskellige biokemiske mekanismer* I eksperimenterne tillader ikke at afgøre, *om en af disse histaminliberatorer* frigør histamin ved at udløse den samme biokemiske *mekanisme* som antigen-antistofreaktionen

Forfatteren har undersøgt antirheumatiske stoffers indflydelse på den anafylaktiske histaminfrigørelse hos rotten ved *in vivo-in vitro* eksperimenter og ved *in vitro* eksperimenter. Både steroide og non-steroide antirheumatika hæmmer histaminfrigørelsen i begge typer af eksperimenter Kun klinisk potente antirheumatika som glukokortikoider, natrium aurotiosulfat og fenylbutazon virker hæmmende på histaminfrigørelsen i *in vivo-in vitro* experimenterne I *in vitro* experimenterne opnås der tillige en hæmmende effekt af mindre potente antirheumatika som natrium salicylat, natrium-γ-resorcylat og amidopyrin, forudsat at disse stoffer anvendes i høje koncentrationer Sådanne høje koncentrationer kan ikke opnås *in vivo* med en rimelig dosering. Rækkefølgen i stoffernes potens er den samme i begge typer af eksperimenter nemlig natrium aurotiosulfat > hydrokortison > fenylbutazon > salicylat og amidopyrin. Natrium aurotiosulfat afviger dog derved, at det kun virker *in vivo*

I *in vivo-in vitro* eksperimenterne skyldes den hæmmende effekt forskellige mekanismer alt efter i hvilket tidsrum af sensibiliseringsperioden, der doseres med antirheumatisk stof *Hydrokortison* hæmmer således den anafylaktiske histaminfrigørelse både når det doseres i begyndelsen og mod slutningen af sensibiliseringsperioden I den første del af perioden beror mekanismen på en hæmmet dannelse af mastcellesensibiliserende antistof I slutningen af perioden skyldes den en hæmning af antigen-antistof reaktionen og/eller en hæmning af den biokemiske mekanisme *Fenylbutazon* og *natrium aurotiosulfat* hæmmer kun den anafylaktiske histaminfrigørelse ved behandling i slutningen af sensibiliseringsperioden Mekanismen er en hæmning af antigen-antistof reaktionen og/eller en hæmning af den biokemiske mekanisme Behandling med hydrokortison, fenylbutazon og natrium aurotiosulfat forud for sensibiliseringsperioden har ingen indflydelse på histaminfrigørelsen

I *in vitro* eksperimenterne hæmmes den anafylaktiske histaminfrigørelse af hydrokortison, fenylbutazon, natrium salicylat, natrium-γ-resorcylat og amidopyrin. Mekanismen beror på en hæmning af antigen-antistof reaktionen og/eller en hæmning af den biokemiske mekanisme

Ved hjælp af *in vivo-in vitro* eksperimenterne er det muligt at skelne mellem mekanismerne (a), en hæmning af antistofdannelsen, og (b) en hæmning af antigen-antistof reaktionen, og/eller en hæmning af den biokemiske mekanisme I modsætning til dette kan *in vitro* eksperimenterne kun afsløre en mekanisme beroende på (b)

Kapitel 5

I nærværende arbejde er der udviklet en metode til bedømmelse af anafylaktisk histaminfrigørelse *in vitro* hos rotten Det er godtgjort, at metoden tilfredsstiller de krav, som må stilles til en sådan bedømmelse

Forfatteren har vist, at visse antirheumatiske stoffer, både steroider og non steroider, hæmmer den anafylaktiske histaminfrigørelse Virkningsmekanismen for denne hæmmende effekt er undersøgt Den skyldes forskellige mekanismer, afhængig af i hvilket tidsrum af sensibiliseringsperioden antirheumatisk stof administreres

Artsforskelle samt den almene gyldighed af de opnåede konklusioner diskuteres

Forfatteren har undersøgt antirheumatiske stoffers indflydelse på den anafylaktiske histaminfrigørelse hos rotten ved *in vivo-in vitro* eksperimenter og ved *in vitro* eksperimenter Både steroide og non steroide antirheumatika hæmmer histaminfrigørelsen i begge typer af eksperimenter Kun klinisk potente antirheumatika som glukokortikoider, natrium aurotiosulfat og fenylbutazon virker hæmmende på histaminfrigørelsen i *in vivo in vitro* eksperimenterne I *in vitro* eksperimenterne opnås der tillige en hæmmende effekt af mindre potente antirheumatika som natrium salicylat, natrium-γ-resorcylat og amidopyrin, forudsat at disse stoffer anvendes i høje koncentrationer Sådanne høje koncentrationer kan ikke opnås *in vivo* med en rimelig dosering Rækkefølgen i stoffernes potens er den samme i begge typer af eksperimenter nemlig natrium aurotiosulfat > hydrokortison > fenylbutazon > salicylat og amidopyrin Natrium aurotiosulfat afviger dog derved, at det kun virker *in vivo*

I *in vivo-in vitro* eksperimenterne skyldes den hæmmende effekt forskellige mekanismer alt efter i hvilket tidsrum af sensibiliseringsperioden, der doseres med antirheumatisk stof *Hydrokortison* hæmmer således den anafylaktiske histaminfrigørelse både når det doseres i begyndelsen og mod slutningen af sensibiliseringsperioden I den første del af perioden beror mekanismen på en hæmmet dannelse af mastcellesensibiliserende antistof I slutningen af perioden skyldes den en hæmning af antigen-antistof reaktionen og/eller en hæmning af den biokemiske mekanisme *Fenylbutazon* og *natrium aurotiosulfat* hæmmer kun den anafylaktiske histaminfrigørelse ved behandling i slutningen af sensibiliseringsperioden Mekanismen er en hæmning af antigen-antistof reaktionen og/eller en hæmning af den biokemiske mekanisme Behandling med hydrokortison, fenylbutazon og natrium aurotiosulfat forud for sensibiliseringsperioden har ingen indflydelse på histaminfrigørelsen

I *in vitro* eksperimenterne hæmmes den anafylaktiske histaminfrigørelse af hydrokortison, fenylbutazon, natrium salicylat, natrium-γ-resorcylat og amidopyrin Mekanismen beror på en hæmning af antigen-antistof reaktionen og/eller en hæmning af den biokemiske mekanisme

Ved hjælp af *in vivo in vitro* eksperimenterne er det muligt at skelne mellem mekanismerne (a), en hæmning af antistofdannelsen og (b) en hæmning af antigen antistof reaktionen, og/eller en hæmning af den biokemiske mekanisme I modsætning til dette kan *in vitro* eksperimenterne kun afsløre en mekanisme beroende på (b)

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By

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Abstract

The sea urchin egg offers unique properties for experimental work on the cellular and embryologic level The developmental stages are well suited for the study of teratogenic effects exerted by drugs The development of the sea urchin egg is holoblastic and resembles the early development of the human ovum considerably The gametes and embryos of the regular sea urchin species *Paracentrotus lividus* and *Psammechinus microtuberculatus* have been used in a study of the teratogenic effects of chloramphenicol, nicotine, chlorpromazine, imipramine and thalidomide All the substances tested were found to influence the fertilization, cleavage and differentiation of the larvae The effects of the substances on the cellular and embryonic level are described and the possible mechanism of action is discussed

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The sea urchin egg offers unique properties for experimental work on the cellular and embryologic level The developmental stages are well suited for the study of teratogenic effects exerted by drugs The development of the sea urchin egg is holoblastic and resembles the early development of the human ovum considerably The gametes and embryos of the regular sea urchin species *Paracentrotus lividus* and *Psammechinus microtuberculatus* have been used in a study of the teratogenic effects of chloramphenicol, nicotine, chlorpromazine, imipramine and thalidomide All the substances tested were found to influence the fertilization, cleavage and differentiation of the larvae. The effects of the substances on the cellular and embryonic level are described and the possible mechanism of action is discussed

During the past decade the interest in substances inflicting malformations or general injuries in living organisms has gained an increasing interest. The extensive use of biocides and their repercussions on living organisms of immediate economic interest and on man created an extending research in this field Of particular importance was, however, the discovery of the teratogenic properties of the sedative hypnotic drug thalidomide. In spite of the elaborate pharmacological methods applied in testing drugs before their adoption in clinical use thalidomide, if administered during pregnancy, was ascertained to cause congenital malformations in man. This was a surprise since thalidomide was regarded as a particularly safe drug and even the intake of large overdoses did not seem to cause any persisting ill effects There is, however, some confusion about the teratogenic effect of thalidomide and its mode of action is still obscure

In the present investigation a number of substances with known or suspected teratogenic effect in man were studied on the cellular and embryonic level As material served the gametes and larvae from the regular sea urchin *Paracentrotus lividus (LAMARCK)*, which is the classic material for the study of fertilization, cleavage and development Few organisms have been subjected to a more conclusive experimental work, and the main advantage with this material is that every ripe female delivers several millions of fairly uniform transparent eggs, particularly suited for microscopic examination in the living state Subsequent to insemination the eggs develop within the course of a few days into self maintaining larvae Cleavage and organogenesis are also easy to observe and even minor changes taking place after the addition of an active drug can be registered in the microscope

The fact that the cleavage and embryogenesis are so well investigated also makes it possible to study the specific effect on e g the ectoderm or the endoderm of a certain substance The short period of time necessary for the embryo to reach the self maintaining pluteus stage also minimizes the influence of external experimental factors

Material and Methods

The experiments were carried out at Stazione Zoologica, Naples, during 1969–1971 As was already mentioned the eggs and spermatozoa of *Paracentrotus lividus* served as material, but in some experiments the gametes of *Psammechinus microtuberculatus (DE BLAINVILLE)* were also utilized Most of the experiments were carried out at temperatures of 18–20°C The rate of development is influenced by temperature but the water of the Gulf of Naples is remarkably constant with regard both to temperature and salinity Since there are slight seasonal changes in temperature the experimental conditions were always closely adapted to the conditions prevailing in the sea

The animals are opened by a section through the equator of the test and the ovaries are placed in filtered sea water in which the eggs are spontaneously shed Eggs and ovaries are separated by filtering through bolting silk of appropriate gauge The eggs are washed by sedimentation in filtered sea water in order to remove the contaminating ovarial and perivisceral fluids Every female delivers 8–10 million eggs and consequently the same egg population is sufficient for the simultaneous testing even of several substances or for several different experiments at a time The testes are placed on bolting silk and the sperm is kept in the "dry" undiluted condition until the moment of insemination At insemination the concentration of egg cells is kept at about 1000/ml and that of the sperm is about 10^5/ml

The determination of the fertilization rate was carried out according to the method of HAGSTROM & HAGSTROM (1954, 1959) in which the fertilization is interrupted at for instance 5, 10, 15 etc seconds after insemination by the addition of sodium lauryl sulphate By this fertilization rate method it is possible to determine the effect of a certain substance on the eggs, on the spermatozoa and on the interaction of the gametes Moreover, it is also possible to study the influence of a substance on the life span of eggs and spermatozoa

The chromosomes are very small in sea urchins and in most species the diploid number is 35–40 Consequently it is not easy to study changes in the chromosomes themselves but changes in their distribution and movements during mitosis and in their arrangement in metaphase and anaphase are possible to observe Likewise the size of the interphase nucleus and the spindle in cells treated with an active substance are possible to determine For the study of the chromosomes the larvae were fixed in Carnoy s fluid transferred to absolute ethanol and later stained in Belling s aceto-carmine

The substances to be tested were applied in consecutive treatments with a duration of as a rule 3 hours After this period part of the treated larvae were washed and transferred to filtered sea water whereas part of the larvae were left in the presence of the substance until the end of the experiment

Every third hour a new lot of larvae from the control was subjected to treatment in several concentrations of the substance The concentrations ranged from the lowest giving any effect, in the present experiments as a rule 10^{-8} molar, to 10^{-4} M. In a few experiments the maximum concentration used was 10^{-4} M, which in most cases inflicts a rapid cytolysis of the embryo

To give even a superficial account of the different developmental stages of the sea urchin egg would be beyond the scope of this paper Therefore, the reader is referred to the books of LEHMANN (1945), v UBISCH (1953) and KUHN (1965) which give a general outline of sea urchin embryology

The following schedule gives the different developmental stages mentioned in this investigation and the approximate interval of time from insemination up to the stage in question for the *Paracentrotus* egg at the temperature of 19°C.

Stage	Time
4-cell	100 minutes
16-cell	180 –"–
32-cell	220 –"–
64 cell	260 –"–
128-cell	300 –"–
Movements inside the membrane	8 hours
Hatching (about 1000 cells)	10 –"–
Primary mesenchyme	12 –"–
Start of invagination	15 –"–
Gastrula with secondary mesenchyme	20 –"–
Prism	24 –"–
Pluteus, short arms	36 –"–
Pluteus, long arms	48 –"–

For electron microscopic studies larvae were fixed in 2–4 per cent osmium tetroxide in sea water or in 2 5–5 per cent glutaraldehyde in sea water, followed by postfixation in osmium tetroxide After washing in sea water the larvae were dehydrated in ethanol followed by acetone before embedding in araldite (Durcupan ACM from Fluka) Sectioning was performed with a *LKB Ultrotome III* and after staining with lead (REYNOLDS 1963) the sections were examined with a Siemens Elmiskop I

Results

Chloramphenicol This broad spectrum antibiotic, which has seen considerable clinical use has also been used previously in experiments with sea urchin material (LALLIER 1962, HORSTADIUS 1963)

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treatment with chloramphenicol is performed from the 500-cell stage the suppressing action on ciliation and hatching is still more accentuated

A treatment in 10^{-5} M for 3 hours before or after hatching causes a delay in the immigration of the primary mesenchyme and the invagination of the gut. If the concentration of chloramphenicol exceeds 10 ° M the invagination is inhibited often resulting in a persisting blastula stage with no subsequent differentiation. These larvae often show an increased ciliation and conform with the classic description of animalized larvae (see e g RUNNSTRÖM 1954) The skeleton becomes also abnormal which certainly is correlated with the aberrant behaviour of the primary mesenchyme Larvae treated during the cleavage stages in moderate (10^{-6} M) concentrations of chloramphenicol for 3 hours also exhibited clear disturbances of the skeleton in the pluteus stage about 45 hours after the treatment had been interrupted (cf Figs 1 and 2) If treatments of 3 hours duration are applied later on the general effect of



Fig 2 Chloramphenicol 3 plutei after treatment The skeleton and also the intestine are abnormal 500×

10^{-3} M and short- and long time treatments have been applied simultaneously using eggs from one female in the same experiment

When treatment is started during the cleavage stages, for instance from 32 cell, and the concentration of chloramphenicol is kept at about 10^{-5} M there is a considerable decrease in the rate of cleavage After a treatment for 3 hours the control is one cell division ahead of the larvae subjected to chloramphenicol At this stage, about 7 hours after fertilization, the control larvae show the first signs of ciliation and soon they start to move slowly inside the fertilization membrane Both ciliation and hatching are strongly delayed by chloramphenicol in concentrations above 10^{-6} M The delay in hatching amounts to 3–4 hours and in 10^{-4} M hatching is inhibited to a fairly high degree, resulting in strongly abnormal development inside the membrane It is therefore to be concluded that chloramphenicol inhibits the formation or the action of the proteolytic hatching enzyme (cf LUNDBLAD 1950) If the

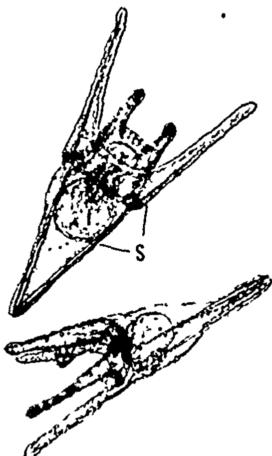


Fig 1 2 control plutei with long arms the upper seen in ventral view the lower in lateral view Note the skeleton (S) and the intestine (I) 500×

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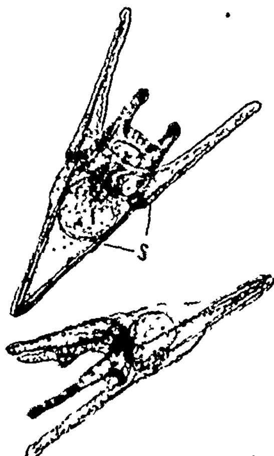


Fig 1 2 control plutei with long arms the upper seen in ventral view, the lower in lateral view Note the skeleton (S) and the intestine (I) 500×

lasting for several days, after which the embryo is destructed by dark cytolysis

The negative effect on hatching exerted by nicotine is achieved also when the treatment is started a short time before the normal breakdown of the fertilization membrane If the treated unhatched larvae are thoroughly washed and transferred to sea water there is no hatching. A tentative explanation of this latter effect is that the hatching enzyme, or the production of this enzyme, is inhibited by nicotine These embryos disintegrate rapidly and the separated cells inside the membrane were observed to divide before cytolysing within 20–24 hours

As already mentioned, embryos treated during the cleavage stages in fairly moderate concentrations of nicotine (10^{-6}–10^{-3} M) in most cases do *not* develop beyond the blastula stage If the larvae have hatched or if nicotine is added after hatching these larvae fail to differentiate an intestine and a skeleton The ciliation becomes increased and the larvae are "animalized" according to the terminology used by sea urchin embryologists (cf RUNNSTRÖM 1954) This stage of development may prevail for two or three days after which there is a rapid immigration of cells from the blastula wall filling the blastocoel and thereupon cytolysis and death follow However, if these nicotine treated 'animalized' blastulae were transferred to pure sea water the blastomeres became separated and a cell suspension was formed The cells were able to divide and they also had the ability to reaggregate with each other and with intact larvae.

Short-time treatments with nicotine during and after the invagination of the gut and after the formation of the primary skeleton also give negative effects on the development though to a lesser extent than in material treated during the cleavage stages

Summarizing the effects of nicotine on cleavage and differentiation it is apparent that there is a general repression The fact that blastulae without, or with highly reduced, gut and skeleton are formed is not in itself an indication of a specific action on the endoderm or the mesoderm It just follows as a consequence of the application of nicotine during the interval of time when these structures are normally formed and differentiated Treatment with nicotine in sufficient concentrations during the early cleavage likewise checks the further development more or less completely, which is in support of the view that nicotine acts as a general inhibitor of the cell The effect of nicotine is stronger during cleavage than during the gastrula stage and the explanation of this phenomenon was found in our studies of the effect of nicotine on the mitotic nucleus

Cytological studies of the effect of nicotine In the study of polyspermy it was indicated that nicotine interferes with mitosis (HAGSTRÖM & ALLEN 1956) and the present experiments strongly substantiated this view Fixations were regularly made from the different developmental stages in order to study the

chloramphenicol decreases but there still are specific injuries to the larvae even in concentrations of 10^{-6}–10^{-7} M

So far the results described have reference to short-time treatments in low to medium concentrations of chloramphenicol (i e 10^{-8}–10^{-5} M) In experiments with long-time treatment started during the cleavage stages and proceeding until the control reached the pluteus stage, the negative effects increased considerably Even in concentrations of 10^{-7}–10^{-6} M chloramphenicol the endoderm is severely damaged and the differentiation of the skeleton is highly pathological or even lacking Often the larvae only reach a stage of slowly swimming blastulae The effect of long-time treatment in chloramphenicol is also characterized by an apparent loosening of the cell contacts in the vegetal region, i e the area in which the intestine is formed In concentrations of 10^{-3}–10^{-4} M the cleavage is rapidly checked and after 30–40 hours there is a general cytolysis

As was already stressed cleavage is delayed in the presence of chloramphenicol Studies of the chromosomes in mitosis revealed that the disjunction of the chromosomes is rendered difficult Lagging chromosomes are frequently occurring and at anaphase a few chromosomes were often observed in between the chromosome plates (cf HAGSTROM & LONNING 1967)

Nicotine Already in 1887 HERTWIG & HERTWIG discovered that nicotine interferes with the mechanism which at insemination prevents more than one spermatozoon from fertilizing the egg HAGSTROM & ALLEN (1956) demonstrated that this mechanism is correlated with the cortical reaction and the subsequent formation of the hyaline layer, these results were later confirmed by LONNING (1965) from electron microscopic studies It was shown that nicotine affects the propagated cortical reaction in the surface layers of the egg In sufficient concentrations of nicotine the cortical reaction is stopped completely and as a consequence the hyaline layer formation is defective and there is no protection against supernumerary spermatozoa

Of particular interest in this connection is the observation that pretreatment in nicotine interferes with the nuclear division even if the egg is monospermic

Pretreatment in nicotine does not interfere with the fertilization rate, though the ensuing cleavages are delayed or inhibited Only a few minutes of pretreatment in 10^{-5} M nicotine brings about a clear delay in cleavage and the pretreated eggs may remain undivided while there are 4-cell stages in the control (cf HAGSTROM & ALLEN loc cit)

When nicotine is added after insemination and is present during the early development the retardation of cleavage is accentuated even in concentrations as low as 10^{-8}–10^{-7} M The same statement is valid also for hatching which is inhibited if the concentration is increased to about 10^{-5} M The ensuing development, which takes place inside the fertilization membrane, is retarded and in most cases there is no differentiation beyond an early blastula stage



ture of the gastrula wall n F g 3 N nucleus with nucleolus (Nu) m tochondrion O o l droplet, Y yolk granule 15 000×

rt and long treatments in nicotine The concentrations of nicotine between 10^{-8}–10^{-4} M Thousands of nuclei in different stages of e been observed and the results are uniform though the effect of *more easily studied in the larvae* before hatching due to the con higher mitotic activity during this period (cf. MORGAN 1895) centrations of 10^{-5}–10^{-4} M there is a high number of nuclei in which has been stopped during metaphase The metaphase plates have not le to separate and form a condensed cluster of chromatine in which omosomes are indistinctive In 10^{-6} M and lower concentrations there adual increase in nuclei in which the metaphase plates are separated which some chromosomes are lagging in the plane between the chromo- plates at anaphase Examples of anaphase plates which are contracted lso frequent The facts point to the interpretation that nicotine affects the dle and the movements of the chromosomes The non-disjunction caused *n cotine closely resembles that found after treatment with lithium ions* AGSTROM & LONNING 1967) In lower concentrations of nicotine the laming fect on the movements of the chromosomes is diminished but it is still evi ent in $5\times10^{-}$ M as is also the retardation in cleavage

It was described above how the nicotine treated larvae often stop at a blas tula stage and become filled with mesenchyme *or show a tendency towards a* separation of the blastomeres In the series of ultramicrographs shown here (Figs 3–7) the control was in a late gastrula stage when the fixation was made (Figs 3 4) The larvae seen in Figs. 5–7 which *were fixed at the same* time had been treated during 16 hours from hatching in 5×10^{-5} M nicotine

These larvae were in a blastula stage many of them with apparent separa tion of the blastomeres but they were relatively good swimmers In Fig. 3

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Of particular interest in this connection is the observation that pretreatment in nicotine interferes with the nuclear division even if the egg is monospermic

Pretreatment in nicotine does not interfere with the fertilization rate though the ensuing cleavages are delayed or inhibited Only a few minutes of pretreatment in $10^{-[illegible]}$ M nicotine brings about a clear delay in cleavage and the pretreated eggs may remain undivided while there are 4 cell stages in the control (cf HAGSTROM & ALLEN loc cit)

When nicotine is added after insemination and is present during the early development the retardation of cleavage is accentuated even in concentrations as low as 10^{-8}–$10^{-[illegible]}$ M The same statement is valid also for hatching which is inhibited if the concentration is increased *to about* $10^{-[illegible]}$ *M* The ensuing development which takes place inside the fertilization membrane is retarded and in most cases there is no differentiation beyond an early blastula stage



Fig. 4 Detail picture of the gastrula wall in Fig 3 N nucleus with nucleolus (Nu), M mitochondrion O oil droplet, Y yolk granule 15,000×

effect of short and long treatments in nicotine The concentrations of nicotine have varied between 10^{-8}–10^{-4} M Thousands of nuclei in different stages of mitosis have b[illegible] observed and the results are uniform though the effect of nicotine is mor[illegible] studied in the larvae before hatching due to the considerably higher [illegible] activity during this period (cf. MORGAN 1895)

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Fig 3 Part of a control gastrula with wall and primary mesenchyme cells Two mesenchyme cells form a syncytium (A) and a cytoplasmic bridge (B) connects several cells Empty vesicles (S) are sites of the skeleton C cilium, H hyaline layer covering the outside of the gastrula wall, on the inside a diffuse layer (D) is present When nothing else is mentioned the larvae in the electron micrographs shown were fixed directly in OsO_4 5,000×

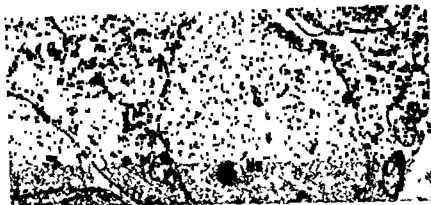


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It was described above how the nicotine treated larvae often stop at a blastula stage and become filled with mesenchyme or show a tendency towards a separation of the blastomeres. In the series of ultramicrographs shown here (Figs. 3–7) the control was in a late gastrula stage when the fixation was made (Figs. 3, 4) The larvae seen in Figs. 5–7, which were fixed at the same time, had been treated during 16 hours from hatching in 5×10^{-5} M nicotine. These larvae were in a blastula stage, many of them with apparent separation of the blastomeres but they were relatively good swimmers In Fig. 3,



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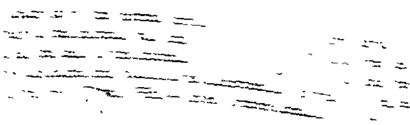




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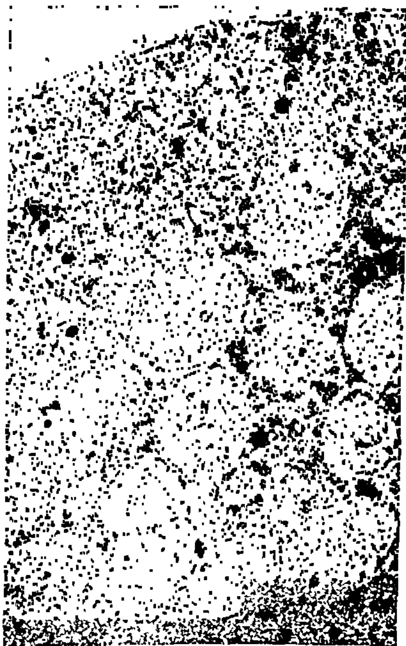


Fig 5 Nicotine Blastula of the same age as the gastrula in Fig 3 treated in 5×10^{-5} M nicotine from hatching during 16 hours Most cell organelles have disappeared N nucleus 5,000×

which shows the normal appearance of a sea urchin larva of this stage, with part of the gastrula wall and several primary mesenchyme cells in the gastrocoel, the cells of the gastrula wall are in close contact and the distribution of mitochondria and yolk is to be described as "normal". A higher magnification of the gastrula wall (Fig 4) gives a more detailed picture of the cytoplasmic



Fig 6 Nicotine A similar larva as in Fig 5 The nuclear membranes are indistinct and between the nuclei vesicles are present which may be rests of the cell borders. 15 000×

components For description of the mesenchyme cells, see thalidomide, pp 41, 42

The ultramicrographs of the treated eggs indicate that nicotine inflicts heavy changes of the cytoplasm and also of the cell membrane, see Fig. 5 In this larva the nuclei are concentrated in the middle of the larva and the cell borders have more or less disappeared resulting in the formation of a large syncytium (cf also Fig. 6 from a similar larva) The structure of the cytoplasm is very loose and both mitochondria and yolk granules are indistinct. This is in agreement with observations from unfertilized eggs treated in nicotine (LÖNNING 1965) The nuclei are likewise very much changed and the nuclear membranes are not continuous but almost seem to be perforated (Fig. 6) The different layers of the nuclear membrane are split and separated and also the nucleoplasm is aberrant. The change in the nuclei is particularly evident in Fig. 7, where there is a phase separation of the nucleoplasm (cf RUNNSTRÖM 1963)

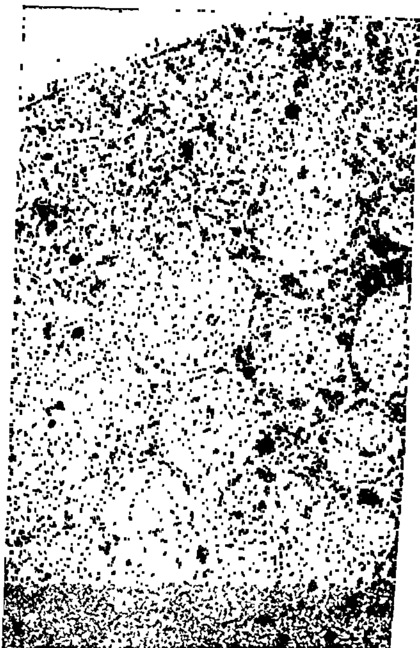


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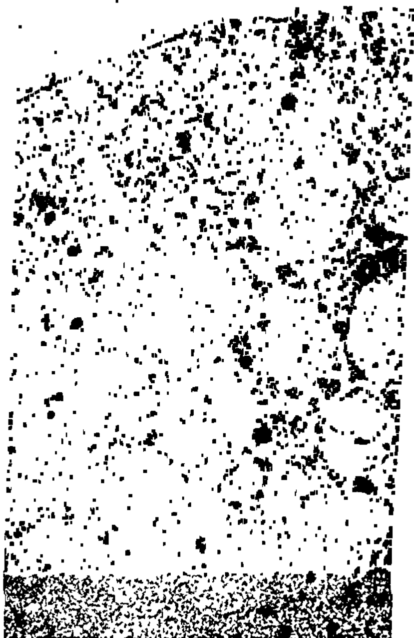


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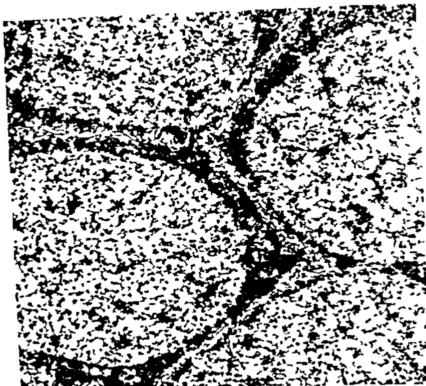


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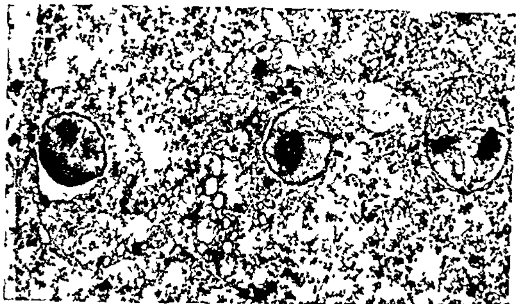


Fig 7 Nicotine Another larva after the same treatment as in Figs 5, 6 Note the three nuclei with condensed nucleoplasm 6 000×

From the ultramicrographs it appears that there is a grave change in the cytoplasm and that the cell membranes and nuclear membranes are severely affected Though the concentration of nicotine was 5×10 ⁵ M our records from the observations of living material describe the effect on the larvae as weak and as already pointed out the swimming capacity was good The culture was alive for 50 hours, the control had then reached the pluteus stage whereas the treated larvae showed definite signs of beginning cytolysis

The effects of nicotine described above appear to have severe implications and we therefore have repeated the experiments during four separate visits to Naples but the results have been congruent In order to settle if these grave cytological changes also take place after a treatment of short duration we started an experimental series in which larvae were treated from hatching in 6×10^{-6} M nicotine, and fixed after 2 4 and 18 hours of treatment The latter fixation showed the same severe changes seen in Figs 5–7 and, if there is a difference, the negative effects were still more accentuated in this experiment

The fixations made 2 and 4 hours after the beginning of the exposure to nicotine resembled each other closely and the disturbances after only 2 hours of treatment were of the same magnitude as those recorded after 4 hours The injuries to cell membranes, nuclei, nuclear membranes, yolk membranes mitochondria and cytoplasm are clearly visible (Figs 8 and 9) and it is also evident that in the outer part of the embryo wall the cell membranes have dissolved more or less completely forming the syncytium common after long time treatment in nicotine (cf Fig 5) The damage is not exclusively confined to



Fig. 8 Control Blastula just after hatching 6,000×

the surface layers, however, since the blastocoel contains an unusual amount of osmium fixed material, which indicates a substantial leakage from the inner parts of the blastomeres It is also evident that the breakdown of the fertilization membrane is very incomplete due to the action of nicotine. From Figs 8 and 9 it is evident that even a fairly short exposure to nicotine at a moderate concentration effects profound disturbances of the cell

The fixation in Figs 5–9 was made in osmium tetroxide Fixations were also made in glutaraldehyde with essentially the same results This latter fixation is, however, leaving the cytoplasm in a less well-preserved condition and the granular components often disintegrate Figs 5–9 therefore give a close representation of the changes in the cell under the experimental conditions described

Chlorpromazine (CPZ) The effect of CPZ on fertilization This substance has very distinct effects on fertilization, cleavage and differentiation. The concentrations tested varied between 10^{-8} and 10^{-4} M.

The effects on fertilization are illustrated by Figure 10 In this fertilization rate experiment the concentrated sperm from the testes was diluted in sea



Fig 9 Nicotine Blastula of the same age as the blastula in Fig 8 treated in 6×10^{-6} M for 4 hours Especially the outer part of the blastula wall is heavily affected Bc blastocoel, F fertilization membrane 6 000×

water or sea water containing chlorpromazine 2 minutes before insemination the concentration of spermatozoa was 10^{5}/ml at insemination, and the concentration of CPZ 10^{-5} M

In Fig 10 curve No 1 represents the control which has a rate of fertilization normal for the concentration of sperm and the temperature of the experiment, 19 5°C In curve No 2 CPZ was added to a final concentration of 10^{-5} M at the moment of insemination Consequently the gametes were not influenced before their interaction but in this case there is a clear retardation in the fertilization rate When the eggs are pretreated for 10 minutes and fer-

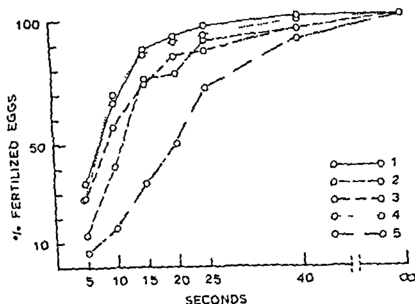


Fig 10 Chlorpromazine Eggs and sperm from *Paracentrotus lividus* Treatment in 10^{-5} M CPZ 1) Control 2) CPZ was added at the moment of insemination 3) The eggs were pretreated for 10 minutes and inseminated in the presence of CPZ 4) The eggs were pretreated for 10 minutes and washed before insemination 5) The spermatozoa were pretreated for 2 minutes and diluted at insemination

tilized in the presence of the substance the resulting fertilization rate, curve No 3 is enhanced as compared with No 2, but slower than in the control and in the following curve No 4 In the latter the eggs were pretreated for 10 minutes and were thoroughly washed in sea water before the insemination *There is hardly any significant effect of CPZ in curve No 4*

In curve No 5 the spermatozoa were diluted in sea water containing 10^{-5} M CPZ *After 2 minutes of treatment the sperm* suspension was used for insemination when the concentration of CPZ was lowered to 10^{-8} M, a concentration which was found to be quite ineffective in experiments of this type From curve No 5 it is obvious that CPZ interferes with the spermatozoa since the fertilization rate is impaired

Thus CPZ interferes with the spermatozoa and their reaction with the eggs whereas the action of this substance on the unfertilized egg is negligible Curve No 5 clearly shows that the spermatozoa are affected, which conclusion is also supported by curves No 2 and 3

The effect of CPZ on cleavage and differentiation Chlorpromazine has a strong effect on cleavage, which becomes retarded or even completely stopped If 16-cell stages are subjected to a concentration of 5×10^{-5} M *cell division is*

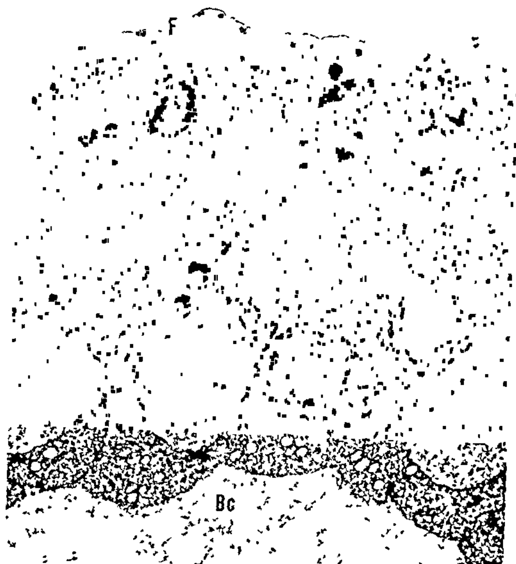


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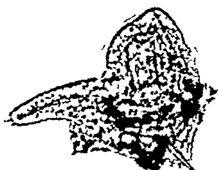


Fig. 13 CPZ Pluteus with destroyed skeleton 500×

integrate An almost complete separation of the blastomeres occurs and a cell suspension is formed within the fertilization membrane in unhatched larvae, cf Fig 11 These cells are not brownish but retain their yellow colour Moreover the separated cells are not in cytolysis since they have the capacity of multiplication The divisions of the separated cells are irregular and deviate in some respects from the normal mitotic pattern As a rule the separated cell first divides into two unequal cells The daughter cells keep contact with each other and 30–40 minutes after their division they again coalesce to what seems to be one single cell After a few minutes, as a rule only 3–5 minutes, the coalesced cells (or syncytium) divide into 3 cells, which in most cases are of unequal size cf Fig. 12 In some cases we have observed that the separated cells display movements. These latter cells are probably descendants of the ciliated ectoderm cells which retain and duplicate their cilia. In many respects the separated cells are reminiscent of the micromeres in their pattern of cleavage (see HAGSTROM & LONNING 1969)

The further development of the larva was also found to be sensitive to the action of chlorpromazine There are, however, different phases of susceptibility during the development Disturbances in differentiation following treatment during the cleavage stages should be compared with the effects achieved in connection with treatment in the later developmental stages when the visible differentiation takes place One generally has to expect a stronger

in most cases stopped before or during the first following cleavage In concentrations of 10^{-5}–10^{-6} M CPZ has also a stopping effect on many of the larvae though the cells may first pass through a few cell divisions The stopped embryos seem to be relatively unchanged for hours and the colour of the larvae may also remain normal, i e slightly yellowish When such embryos are washed and transferred to pure sea water there is a rapid change to a brownish colour and the blastomeres of most larvae begin to cytolyse Even in a concentration of only 10^{-8} M there is a clear delay in the rate of cleavage

The statements above are valid for treatments from the early cleavage stages, preferably the 16 cell stage When the treatment is started somewhat later and the concentration is kept below 10^{-6} M there is still an obvious delay in the cleavage which, however is not stopped completely Such larvae may develop *into relatively advanced stages but there is always a certain* variation and many embryos will after a few hours of treatment start to dis

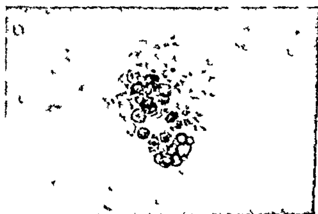


Fig 11 CPZ Cell separation of a larva after treatment 500×

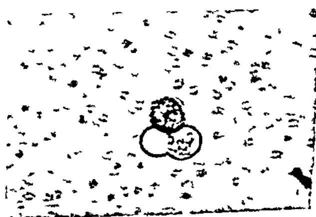


Fig 12 CPZ Three cells formed after cleavage of a separated blastomere 1500×

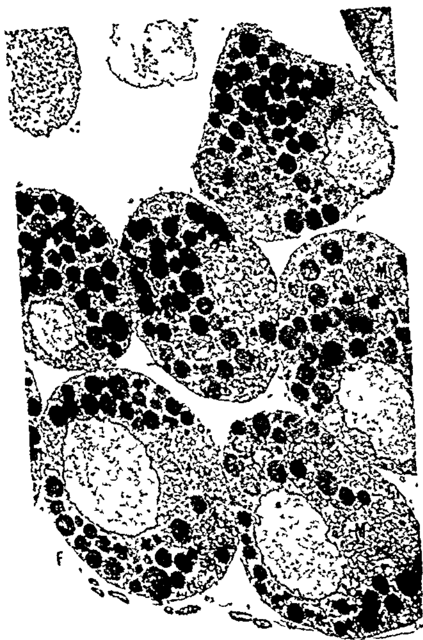


Fig. 14. [illegible] Separated blastomeres within the fertilization membrane (F) after treatment in [illegible] M CP[illegible] from 250-cell for 22 hours. Note the very distinct yolk granules (Y) and concentrations of mitochondria (M). 6,000×

manifestation of a substance if the treatment is started in the early cleavage stages than in the later stages of development when organogenesis has already started (cf HAGSTROM & LONNING 1967) The possibility that a relatively weak treatment in low concentration and of short duration, applied in the cleavage stages, could be equal to a stronger and more extended one in the later developmental stages has been tested in our experiments If the substance used has a direct or indirect effect on the genetic material it is not very likely that this combined time dose effect will materialize

If the treatment is started in the cleavage stages *from 16- or 32-cell* and the concentration *is about* 5×10^{-7} M the resulting larvae show severe injuries to the skeleton, provided the treatment is going on up to the pluteus stage in the control Such larvae do reach a pluteus stage but there are deformations of the skeleton which is also quantitatively somewhat reduced These injuries are mainly concentrated to the oral arms and the oral field, cf Fig 13 The larvae are vital and their swimming capacity is not much impaired

In the higher concentration of 5×10^{-6} M the negative effect is *much more pronounced* Already after 3 hours of treatment the cleavage was stopped in some of the embryos After 5 hours treatment about 25 per cent of the larvae showed evidence of beginning cytolysis At the same time there were 50 per cent hatched larvae in the control whereas in the treated batch the best embryos only comprised about 500 cells From this it is evident that treatment in CPZ brings about a less uniform development and that the variation in morphological stages within the culture is increased After 20 hours the control had reached the prism stage whereas the best larvae in 5×10^{-6} M CPZ were in a blastula stage in which the blastocoel was filled with mesenchyme Most of the larvae, however, were disintegrating and the blastomeres had become separated from each other As already indicated CPZ acts inhibiting on hatching and in some instances this cell separation had taken place within the fertilization membrane There were also numbers of free blastomeres from disintegrated hatched larvae These cells were capable of the same type of irregular cell division which was already described above

When the same concentrations were used in short consecutive treatments, which were interrupted by subsequent washing in and transfer to pure sea water the effect registered was considerably weaker

In 5×10^{-6} M CPZ started at the 16 cell stage and interrupted after a treatment for 3 hours the results were in some respects surprising There was a tendency towards cytolysis and there were also embryos stopped already in the 16 cell stage After 3 hours of treatment and transfer to sea water the 'best' larvae admittedly reached a more advanced final stage of development since a few gastrulae with redu[illegible] formed Most of the surviving embryos only reache[illegible]



Fig 14 CPZ Separated blastomeres within the fertilization membrane (F) after treatment in 5×10^{-5} M CPZ from 250-cell for 22 hours. Note the very distinct yolk granules (Y) and concentrations of mitochondria (M) 6,000×

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In 5×10^{-6} M CPZ started at the 16-cell stage and interrupted after a treatment for 3 hours the results were in some respects surprising There was a tendency towards cytolysis and there were also embryos stopped already in the 16 cell stage After 3 hours of treatment and transfer to sea water the "best" larvae admittedly reached a more advanced final stage of development since a few gastrulae with reduced gut and skeleton were formed Most of the surviving embryos only reached the blastula stage There was no increase in



Fig 14 CPZ Separated blastomeres within the fertilization membrane (F) after treatment in 5×10^{-5} M CPZ from 250-cell for 22 hours Note the very distinct yolk granules (Y) and concentrations of mitochondria (M) 6,000×

the number of mesenchyme cells Both gastrulae and blastulae were strongly ciliated and good swimmers This refers to the situation 48 hours after fertilization and 42 hours after the treatment in CPZ was finished

When the 3 hours treatment described above was interrupted a new batch of eggs was subjected to the same concentration of CPZ After the treatment had been interrupted at 9 hours the ensuing development was mainly following the same schedule as the preceding treatment The "best" larvae, however, reached a stage which was a little more advanced and there were some plutei with very distorted skeletons but proportionally less injury to the intestine Also in this case there were several abnormal intermediate stages, and a certain proportion of the larvae in which the cells became separated

In experiments where the consecutive treatments were started 9–15 hours after insemination the effect was still more diminished These results indicate that the negative effect of CPZ decreases during development and that the cleavage stages are more susceptible to the action of CPZ In a parallel experiment with 5×10^{-7} M CPZ the results were essentially the same though the response was weaker

Cytological studies of the effect of CPZ The possible effect of CPZ on the nuclei and chromosomes was investigated in material fixed in Carnoy's fluid and stained in aceto-carmine An extensive number of embryos in various stages has been examined but so far no disturbances affecting the chromosomes have been detected As already pointed out mitosis is rapidly brought to a standstill in appropriate concentrations of CPZ The nuclei are already stopped after less than 30 minutes exposure in interphase The nuclei are small, probably somewhat contracted, and display a low stainability in aceto-carmine

In order to get an insight in the morphological changes following treatment with CPZ fixations for electron microscopy were also made, using material from the experiments described above For reference to the normal ultrastructure of the sea urchin embryo, see Fig 3, which shows part of a gastrula stage with wall and primary mesenchyme cells, and Fig 4 for details of the cytoplasm In Fig 14 a larva of the same age is shown, which had been treated in 5×10^{-5} M CPZ from 250-cell until a late blastula which had not hatched This is a rather strong treatment though it was started at a relatively late cleavage stage Fig 14 shows the fertilization membrane (F) which surrounds a number of cells in the blastula wall The cells are very loosely attached to each other and some of them are completely separated from their neighbours The size of the cells which is strikingly uniform, is considerably larger than the cells seen in Fig 3, whereas the nuclei are relatively small This indicates that the treated cells have not passed through so many cell divisions as those of the control and it also substantiates the light microscopic finding that the interphase nuclei of treated cells are contracted



Fig. 15 CPZ A concentration of mitochondria and distinct yolk granules (Y) after the same treatment as in Fig 14 30 000×

There are also other differences between the treated cells and the controls The *hyaline layer clearly visible in Fig* 3 (H) is almost completely lacking after treatment in CPZ The hyaline layer is formed subsequent to the cortical reaction at fertilization and serves as a cover which keeps the cells of the embryo together CPZ acts dissolving on the hyaline layer and Fig 14 indicates that the apparent cell separation is correlated with this dissolution The nuclear membranes are also aberrant which may indicate that there is a change in the osmotic properties of this membrane After treatment in CPZ the yolk *granules (Y) are very distinct and it seems as if the mitochondria are con*centrated to certain restricted parts of the cytoplasm (Figs 14, 15) The mitochondria are often more elongated after treatment in CPZ and the number of cristae seems to become increased (Fig. 15) After treatment in CPZ lysosomes sites of lytic activities, also become frequent.

As was already stated treatment with chlor*promazine in the early cleavage* stages effects rapid inhibition of mitosis Since the changes in nucleus and *cytoplasm are clearly visible in the* phase contrast microscope it is possible to make a comparative study with the electron microscope Fig 16a shows a 4-cell stage photographed with phase contrast microscope The larva is stopped by the addition of 10^{-4} M CPZ at the 4-cell stage and has been treated for 16 hours Already at this low magnification it is possible to state that the larva is se*verely affected, with shrunken, separated b*lastomeres In-



Fig 16 CPZ Larvae stopped after treatment in 10^{-4} M from the 4 cell stage Fig 16a photographed with phase contrast microscope, magn 500× Fig 16b the ultrastructure of a similar larva Most cell organelles are indistinct *O oil droplet*, *Y former yolk granule*, *F fertilization membrane* 6 000×



Fig 17 CPZ The outer layers of a larva treated as in Fig 16 F fertilization membrane Note the inner membrane which at several places can be seen to consist of a double layer 30,000×

side and close to the fertilization membrane a thin, rather indistinct membrane is observed

Fig 16b shows the ultrastructure of part of a 4-cell stage from the same batch of larvae as Fig 16a. The cytoplasm is heavily affected the yolk granules have become broken up and mitochondria can no longer be distinguished Only some oil droplets are distinct in the cytoplasm Also the cell membranes have become broken up and between the separated blastomeres there is a homogeneous, rather dense substance The picture reminds of the "phase separation" described by RUNNSTRÖM (1963) The cortex layers which keep the blastomeres together have also been transformed in a very peculiar manner Also in this region the homogeneous substance is present, and inside the fertilization membrane (F) the indistinct membrane from Fig 16a is observed, for a higher magnification see Fig 17. This membrane, which often is seen as a double layer, is difficult to identify, but it may correspond to the hyaline layer of the normal larva. This is a variant, and a more far-reaching one, of the changes seen in Fig. 14. Both these pictures indicate the severe action of chlorpromazine on the cell surfaces and the connections between the cells The unusually high number of oil droplets seen in Fig 16b is also an indication of the fundamental rearrangement of the cytoplasm following treatment in CPZ.

Imipramine The active principle of "Tofranil" (from Geigy) is imipramine In 1969 the action of Tofranil-solution was investigated and the drug was found to exert severe teratogenic effects on the embryo (HAGSTRÖM 1969) Cell division was rapidly brought to a standstill and hatching of the larva was inhibited completely even in a concentration of 10^{-6} M It is surprising that



Fig 16 CPZ Larvae stopped after treatment in 10^{-4} M from the 4-cell stage Fig 16a photographed with phase contrast microscope magn 500× Fig 16b the ultrastructure of a similar larva Most cell organelles are indistinct O oil droplet Y former yolk granule F fertilization membrane 6 000×



Fig 19 Imipramine A well developed rough surfaced endoplasmic reticulum after treatment in 5×10^{-5} M from the 16-cell stage for 3 hours Fixation 16 hours later when the control had reached the gastrula stage (cf Fig 3) This larva was fixed in glutaraldehyde 60 000×

Tofranil in clinical use has not been reported to give any more adverse side-effects.

Chlorpromazine and imipramine are from the chemical point of view closely related to each other Despite the fact that their pharmacological effect is different it is to be expected that their action on the cell in cleavage and differentiation will be rather similar This was also found to be the case and the similarities in the influence on differentiation are so close that we refrain from giving a survey of the development but refer to the description given above for chlorpromazine

There are however also some differences in action between these two substances When the same molar concentrations are applied of CPZ and imipramine and eggs from the same female are used imipramine has a definitely stronger effect on cleavage and cell separation Thus the concentration necessary for stopping cleavage completely seems to be 10–15 times lower in imipramine than in CPZ Also the disaggregation of the blastomeres occurs much more rapidly in imipramine For instance treatment in 5×10^{-6}–10^{-5} M imipramine started at 8–16-cell resulted in about total cell separation at the 16-cell stage which means that the effect was immediate Consequently the quantitative effect of imipramine is stronger though qualitatively the action is to be compared with that of CPZ

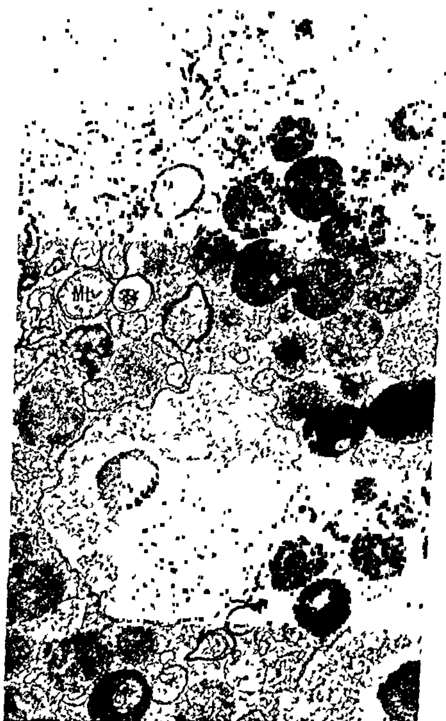


Fig 18 Imipramine A syncytium formed after treatment in 10^{-6} *M* from the 16-cell stage *Note the nucleoli surrounded by a less dense corona, the distinct yolk granules* and mitochondria which seem to become broken down lytically (ML) 13 500×

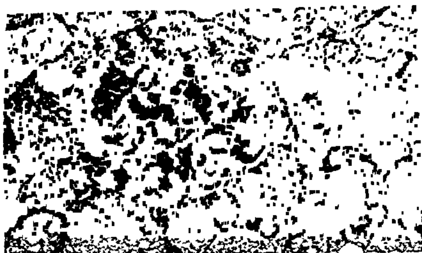


Fig. 20 Imipramine A large lysosome (L) in a larva treated as in *Fig 19 Note the* distinct yolk granules 15 000×

spermatozoon Thalidomide is not very soluble in sea water and the maximum solubility at 20°C is about 10^{-4} M As was found when testing the activity of the drug the solutions in sea water are not stable and if stored at +5°C a solution of 10^{-4} M becomes inactive within the course of 2–3 hours The aged solutions have little or no influence on the sea urchin larva

The effect of thalidomide on fertilization The action of thalidomide on the gametes and on fertilization was tested in experiments according to the fertilization rate method referred to above (*HAGSTRÖM & HAGSTRÖM 1959*) *Fig* 22 illustrates an experiment in which sperm and eggs were subjected to thalidomide treatment either prior to or during insemination or both

The concentration of thalidomide was in this experiment 2×10^{-6} M It is evident that thalidomide when present at insemination has a slight retarding effect on fertilization (curve No 2), and this is somewhat more accentuated if the eggs have been pretreated for 10 minutes before the addition of the sperm (curve No 3) Pretreatment of the eggs followed by washing in and transfer to sea water before insemination has, however, no deleterious influence on the course of fertilization (curve No 4) We also pretreated the sperm and used the spermatozoa for insemination The fertilization rate was not impaired (see curve No 5) The development of the eggs represented by the last point in the curves No 1–5 was also studied and we will return to the results below

Other concentrations of thalidomide were also tested in fertilization rate experiments but no impairing effect was registered Surprisingly enough a

When studying the ultrastructure further differences distinguishing the effect of imipramine from that of CPZ were observed. When 128 cell stages were transferred into 5×10^{-6} M imipramine for 3 hours, the cells were found to form syncytia when the larvae were fixed 24 hours after insemination and 18 hours after the treatment was stopped. A similar result was achieved when 16-cell stages were treated continuously for about 24 hours in 10^{-6} M imipramine. The control had reached gastrula when the treated larvae, which were in a pathological blastula stage or had disintegrated, were fixed. Also among these larvae syncytia were found, cf. Fig. 18, which shows part of a large syncytium.

Four interphase nuclei can be seen but no cell membranes can be distinguished between the nuclei. In the nuclei nucleoli occur frequently, surrounded by a less dense corona, they are of a rather aberrant appearance. Very dense and well defined yolk granules are also present in abundance and ribosomes are found in excessive numbers. The rough surfaced endoplasmic reticulum is often well developed in imipramine treated embryos (cf. Fig. 19). This is also found in CPZ but not co frequently. The mitochondria are often severely affected (cf. Fig. 18) and may even be difficult to recognize. Often the mitochondria are isolated from the rest of the cytoplasm by an extra membrane and seem to become broken down lytically. This tendency towards a formation of lysosomes is very common after imipramine treatment (cf. Figs. 18, 20), and this is probably also a reflection of the cell separating and cytolytic action of this substance. The hyaline layer is less affected by imipramine than by CPZ which is somewhat surprising since the cell separating effect of imipramine is much stronger.

We described previously the cleavage pattern of the disaggregated blastomeres obtained with CPZ. The same schedule is in principle valid for the cells separated in imipramine. The nuclei may become lobated and show most irregular cleavages and there is also a budding off of parts of the blastomeres as is indicated in Fig. 21.

Imipramine interferes very strongly with the differentation of the embryo, and the formation of the embryonic organs is suppressed. Moreover, imipramine causes profound changes in the cell membranes, in the nuclei and in the various cytoplasmic organelles.

Thalidomide. Due to the severe consequences of the administration of thalidomide during pregnancy extensive studies using different organisms have been carried out in order to reveal the mechanism producing the foetal abnormalities. As yet there is, however, no conclusive apprehension of the teratogenic effect of thalidomide.

The present experiments were made in order to determine the effect of thalidomide on the different germinal layers of the sea urchin embryo. Experiments were also made to elucidate the direct effect on the egg and the

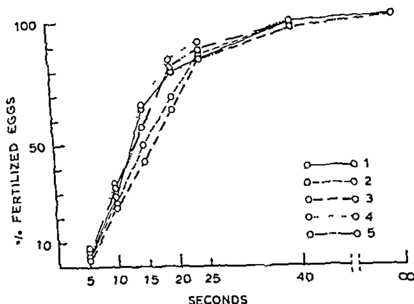


Fig 22 Thalidomide treatment in 2×10^{-6} M [1]) *Control* [2]) Thalidomide added at the moment of insemination [3]) The eggs were pretreated for 10 minutes and inseminated in the presence of the substance [4]) The eggs were pretreated for 10 minutes and washed before insemination [5]) The spermatozoa were pretreated for 2 minutes and diluted at insemination

and there was *no incidence* of polyspermy Thalidomide seems therefore not to exert any harmful effect on fertilization

The effect of thalidomide on cleavage and differentiation Extensive experiments were made on the effect of thalidomide on cleavage, hatching and differentiation The fact, that thalidomide has a restricted stability in solution is of course a disadvantage, but this also implies that a treatment always is of a comparatively short duration. If a prolonged exposure is necessary the drug must be applicated at relatively short intervals In concentrations below 5×10^{-6} M thalidomide has a very slight retarding action on cleavage and hatching If the concentration is increased up to the maximum concentration of about 10^{-4} M the negative effect is somewhat stronger but is still weak The same is true for the differentiation after hatching which up to the gastrula stage is little impaired There is a certain increase in the ciliation and the development of the endoderm may become impaired in connection with an enlargement of the secondary mesenchyme As a whole the effect of thalidomide up to the late gastrula stage can be described as moderate

Therefore, the changes observed at the prism and pluteus stages appear almost dramatic. These changes are in a very predominant way concentrated



Fig 21 Imipramine An irregular cell with lobated nucleus 15 000×

concentration of 10^{-5} M added at the moment of insemination gave a clear improvement in the fertilization rate. The formation of the fertilization membrane and the hyaline layer was quite normal in the presence of thalidomide

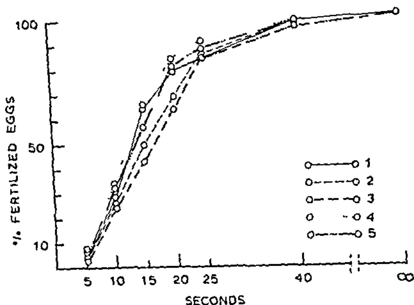


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Therefore the changes observed at the prism and pluteus stages appear almost dramatic These changes are in a very predominant way concentrated

to an abnormal differentiation of the skeleton and the resulting plutei are of very aberrant form There may also be some reduction in size of the skeleton which in extreme cases may be reduced to a single rod (cf Fig 23) This 'pluteus" had a gut with stomodeum and anus and it was a very swift swimmer The same type of pluteus larva has been produced in a number of experiments As already pointed out the damage to the skeleton becomes visible in a relatively late stage of development but in some cases a disarrangement of the skeleton may also be observed in the gastrula stage As a rule the quantitative changes are not so obvious as are the changes in form and spatial extension of the skeleton

The application of thalidomide has been varied thoroughly as regards concentration, time of exposure and stage of development So far no period particularly sensitive to thalidomide has been discovered but the addition of thalidomide after hatching seems to be somewhat less injurious This may be correlated with the considerably reduced mitotic activity after hatching

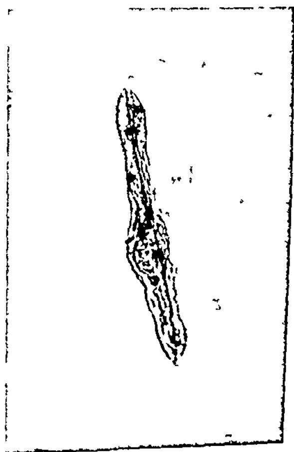


Fig 23 Thalidomide A pluteus with very reduced skeleton cf normal plutei in Fig 1
500×

(cf MORGAN 1895) The concentration of thalidomide clearly influences the extent of injury inflicted on the skeleton and in concentrations above 10^{-7} M there is a graded response of the larvae from very weak *malformations to* severely destructed skeletons

We now return to the fertilization rate experiment illustrated in Fig 22 In this experiment thalidomide acted upon eggs and sperm before *insemination* and the substance was also added at the *moment of insemination* We studied the development from the last point of the curves in which the sperm was not killed by adding lauryl sulphate The control (curve No 1) was quite normal and developed into pluter The larvae of No 2-5 showed all the injuries of the skeleton common after an addition of thalidomide Of particular interest is the fact that the eggs of No 4, which had only been treated for 10 minutes in thalidomide whereupon the eggs had been thoroughly washed in pure sea water before insemination, showed severe damage to the skeleton

In curve No 5 there was no treatment of the eggs, only the spermatozoa were pretreated for 2 minutes before insemination The resulting larvae were reduced in size as compared with the control and they exhibited grave malformations of the skeleton of an order even exceeding that found in the cultures No 2-4 In this experiment thalidomide effected a slight retardation of cleavage *and ensuing development but the abnormalities in differentation* were first observed during late gastrula or prism

The effect of thalidomide in combination with nucleic and amino acids In sea urchins fertilization takes place after the egg has completed both the meiotic divisions In experiments where eggs or spermatozoa have been pretreated in thalidomide before the insemination of the egg is carried out, thalidomide has only acted upon haploid nuclei The question arises whether or not larvae reared from gametes which have both been pretreated become more pathological than embryos reared from one pretreated and one non treated gamete

Since already pretreatment of either egg or sperm evokes severe changes in the differentiation of the skeleton the difference after pretreatment of both gametes is mainly quantitative as could be expected In most experiments a pretreatment of both the gametes inflicts as a rule somewhat more complete disturbances in the skeleton of the pluteus stage *However,* also the concentration of the drug is important and a short exposure of one of the gametes to a high concentration of thalidomide may be equal to pretreatment of both gametes in lower concentrations The important fact is, however, that already a short pretreatment of one of the haploid gametes results in strongly pathological *skeletons 48 hours* after the pretreatment

These experiments intimate that thalidomide inflicts irreversible injuries on a restricted part of the genome of eggs or spermatozoa Another explanation is that thalidomide is stored in the pretreated gametes and is transferred from before insemination through all the cell *divisions, through* hatching, in-

to an abnormal differentiation of the skeleton and the resulting plutei are of very aberrant form. There may also be some reduction in size of the skeleton, which in extreme cases may be reduced to a single rod (cf. Fig. 23). This "pluteus" had a gut with stomodeum and anus and it was a very swift swimmer. The same type of pluteus larva has been produced in a number of experiments. As already pointed out the damage to the skeleton becomes visible in a relatively late stage of development but in some cases a disarrangement of the skeleton may also be observed in the gastrula stage. As a rule the quantitative changes are not so obvious as are the changes in form and spatial extension of the skeleton.

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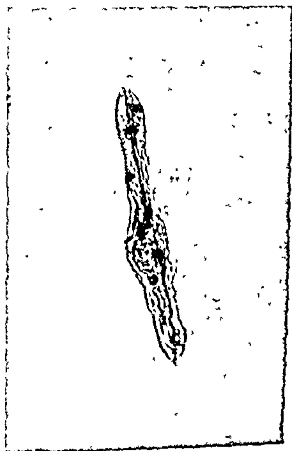


Fig 23 Thalidomide A pluteus with very reduced skeleton, cf normal plutei in Fig 1 500×

whereas the mixtures of thalidomide and nucleic acid never caused any injuries to the skeleton of the plutei which were entirely normal (Figs 24–26)

These experiments clearly demonstrate that nucleic acids react with thalidomide *in vitro* and that the reaction product does not interfere with the differentiation of the larval skeleton (cf Figs 25, 26) The character of the reaction between thalidomide and nucleic acids is still obscure but since DNA and RNA were equally effective in neutralizing the negative action of thalidomide there is presumably no genetic specificity involved

The structure of the thalidomide molecule suggests that a number of other substances might also easily interfere Extensive experiments were carried out with d arginine, d asparagine and d l glutamine The concentrations were equal i e 10^{-5} M of thalidomide was mixed with 10^{-5} M amino acid. These experiments were made simultaneously with the nucleic acid experiments already described and in most cases using the same egg material

The results were entirely uniform and in congruence with those obtained with nucleic acids Arginine, asparagine and glutamine abolished completely the skeleton injuring activity of thalidomide

The solubility of thalidomide in water is fairly low and in many investiga



Fig 25 Thalidomide 10^{-5} M Pretreatment of the eggs for 5 minutes The skeleton is abnormal

vagination etc and first becomes active during the final differentiation of the skeleton Against this assumption speaks strongly the fact that thalidomide is very unstable in sea water and already within the course of a couple of hours the teratogenic effect is abolished completely

In order to settle if thalidomide interacts with nucleic acids we made parallel experimental series in which we subjected the eggs to a short pretreatment in thalidomide or thalidomide + a pure preparation of nucleic acid To ensure grave injuries to the skeleton the concentration of thalidomide was fairly high, 10^{-3} M, whereas the concentration of nucleic acid as a rule was 0 01 mg/ml

Preparations of DNA (B D H and Worthington) and RNA (Eastman Kodak) were used Thalidomide and thalidomide + nucleic acid were mixed with the eggs for 3–5 minutes pretreatment whereupon the eggs were thoroughly washed in sea water before the insemination In other experiments the substances were added immediately after fertilization or at different intervals during the development up to and including the early gastrula stage

In all experiments thalidomide alone inflicted the characteristic severe disturbances in the differentiation of the skeleton already described in detail

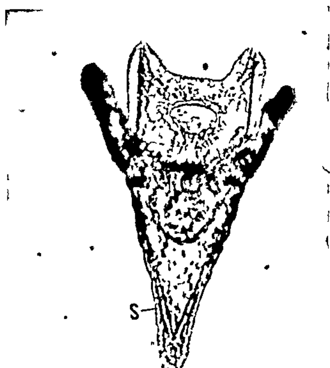


Fig 24 Control pluteus seen in ventral view with a well developed skeleton (S) 500×

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tions the drug has been administered to the experimental animals in a mixture with some as inert regarded substance We have tried two such substances, gum arabic and gum tragacanth The experimental procedure was the same as that used in the experiments with nucleic acids and the concentrations were 10^{-5} M for thalidomide and 0 01–0 1 mg/ml for gum arabic and tragacanth Also in these experiments the effect of thalidomide on the development of the skeleton was neutralized. This result may have some bearing on the varying results obtained with different research organisms It should be pointed out that the pH of sea water is about 8, the pH of the human stomach about 1 5 whereas the pH in the stomach of e g the rabbit is probably intermediate At present we refrain from speculative conclusions, but the stability of a possible reaction product between thalidomide and e g gum tragacanth at different pH should be subjected to a future investigation

Cytological studies of the effect of thalidomide As was already stressed the manifestation of a treatment with thalidomide becomes visible in a relatively late stage of differentiation irrespective of whether the material is treated before fertilization, during the cleavage stages or after hatching Apart from minor disturbances in the rate of cleavage the development proceeds in a rather normal way up to the gastrula stage when the malformations of the



Fig 26 Thalidomide 10^{-5} M + nucleic acid DNA, 0 01 mg/ml Pretreatment of the eggs for 5 minutes Normal skeleton

larval skeleton become apparent. The vitality of the treated larvae is in parity with that of the control and there is no reduction in the swimming capacity. Thalidomide was observed to bring about an increase in the number of the secondary mesenchyme cells whereas no other major change was detected with the phase contrast microscope.

The larval skeleton originates from the primary mesenchyme cells, which immigrate into the blastocoel from the vegetal pole of the embryo. Important contributions about the formation of the skeleton of the sea urchin larva were made by v. UBISCH (1937) and OKAZAKI (1960). We recently demonstrated that the cells of the primary mesenchyme regularly form syncytia, which probably is of fundamental importance to the differentiation of the skeleton (HAGSTROM & LONNING 1969). For an example of the organization of the primary mesenchyme cell see Fig. 3, which as already mentioned shows part of a control gastrula. The empty vesicles (S) are the sites of the skeleton, dissolved during fixation or removed during the sectioning. It is to be observed [illegible] ions in the syncytia are of two somewhat different types.

[illegible]

connects several cells (B) (cf. GIBBINS *et al.* 1969). The cells of the [illegible] wall are in close contact.

Though no deviation in the behaviour of the primary mesenchyme could be detected in the living larva following exposure to thalidomide we prepared material for an electron microscopic study. Fig. 27 shows the vegetal part of a young gastrula. The primary mesenchyme cells with the sites of the skeletal rods (S) are seen as are also two mesenchyme cells forming a syncytium and several cytoplasmic bridges between the cells. At higher magnification (Fig. 28 syncytium in Fig. 27) the nucleus, mitochondria and yolk granules are quite normal and there is no deviation from the appearance of control larvae from the same batch of eggs (cf. Figs. 3, 4). Also the syncytia and the other cellular bridges connecting the mesenchyme cells are normal and we have not been able to establish any difference in ultrastructure between the control and the thalidomide treated larvae.

The concentration of thalidomide was in this experiment as high as 2×10^{-3} M and the treatment was started just before hatching. Thalidomide should therefore have affected the stages preceding the immigration of the primary mesenchyme but regardless of this there are no changes in the ultrastructure which can be ascribed to the action of thalidomide. This is in agreement with the results from the *in vivo* studies and is also in support of the view that thalidomide has no general toxic influence on the embryo and does not affect the primary formation of the skeleton but acts on the mechanism by which the complex final differentiation of the skeleton is achieved.

Penetration studies. It is of particular interest to determine how fast a

tions the drug has been administered to the experimental animals in a mixture with some as inert regarded substance We have tried two such substances, gum arabic and gum tragacanth The experimental procedure was the same as that used in the experiments with nucleic acids and the concentrations were 10^{-5} M for thalidomide and 0 01–0 1 mg/ml for gum arabic and tragacanth Also in these experiments the effect of thalidomide on the development of the skeleton was neutralized. This result may have some bearing on the varying results obtained with different research organisms It should be pointed out that the pH of sea water is about 8, the pH of the human stomach about 1 5 whereas the pH in the stomach of e g the rabbit is probably intermediate At present we refrain from speculative conclusions, but the stability of a possible reaction product between thalidomide and e g gum tragacanth at different pH should be subjected to a future investigation

Cytological studies of the effect of thalidomide As was already stressed the manifestation of a treatment with thalidomide becomes visible in a relatively late stage of differentiation irrespective of whether the material is treated before fertilization, during the cleavage stages or after hatching Apart from minor disturbances in the rate of cleavage the development proceeds in a rather normal way up to the gastrula stage when the malformations of the

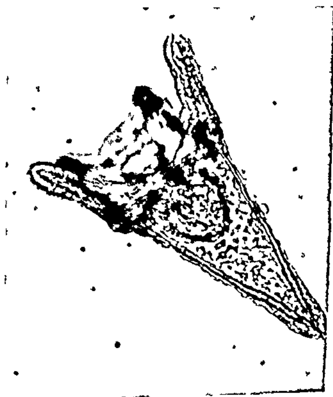


Fig 26 Thalidomide 10^{-5} M + nucleic acid DNA 0 01 mg/ml Pretreatment of the eggs for 5 minutes Normal skeleton

larval skeleton become apparent The vitality of the treated larvae is in parity with that of the control and there is no reduction in the swimming capacity Thalidomide was observed to bring about an increase in the number of the *secondary mesenchyme* cells whereas no other major change was detected with the phase contrast microscope

The larval skeleton originates from the primary mesenchyme cells which ... the blastocoel from the vegetal pole of the embryo Important

made by ...

that the cells of the primary mesenchyme *regularly form syncytia*, which probably is of fundamental importance to the differentiation of the skeleton (HAGSTROM & LONNING 1969) For an example of the organization of the primary mesenchyme cell see Fig 3, which as already mentioned shows part of a control gastrula The empty vesicles (S) are the sites of the skeleton, dissolved during fixation or removed during the sectioning It is to be observed ... in the syncytia are of two somewhat different types

connects several cells (B) (cf GIBBINS *et al* 1969) The cells of ... wall are in close contact.

Though no deviation in the behaviour of the primary mesenchyme could be detected in the living larva following exposure to thalidomide we prepared material for an electron microscopic study Fig. 27 shows the vegetal part of a young gastrula The primary mesenchyme cells with the sites of the skeletal *rods (S)* are seen as are also two mesenchyme cells forming a syncytium and several cytoplasmic bridges between the cells At higher magnification (Fig. 28 syncytium in Fig 27) the nucleus mitochondria and yolk granules are quite normal and there is no deviation from the appearance of control larvae from the same batch of eggs (cf *Figs 3, 4) Also* the syncytia and the other cellular bridges connecting the mesenchyme cells are normal and we have not been able to establish any difference in ultrastructure between the control and the thalidomide treated larvae

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Penetration studies It is of particular interest to determine how fast a



Fig 27 Thalidomide Part of a gastrula with wall and primary mesenchyme cells after treatment in 2×10^{-5} M from hatching during 16 hours Empty vesicles (S) which are sites of the skeleton, syncytium (A) and cellular bridges (B) are seen 5 400×

tested substance penetrates into the cell and if the passage through the covering layers into the interior of the cell effects changes of membranes or cytoplasm

The sea urchin egg is surrounded by a jelly coat consisting mainly of acid mucopolysaccharides This cover gradually dissolves in sea water but serves as a protection of the egg cell against mechanical and chemical influences Most important is, however, that the jelly coat diminishes the incidence of polyspermy and also protects the egg from being activated by heterologous spermatozoa After fertilization the egg forms a new surface cover, the hyaline layer, which mainly consists of mucopolysaccharides Moreover, also during the ensuing development the embryo produces mucus, which covers the outer and inner surfaces of the larva

A fair estimation of the penetration rate and the changes following the exposure of the egg to an active substance is obtained if an egg suspension in a sea water solution of the substance to be tested is centrifuged on the border of an isotonic sucrose solution For control eggs from the same female are simultaneously centrifuged in pure sea water/isotonic sucrose A detailed description of the technique was given by HARVEY (1956)

When control eggs are centrifuged at forces between 4000 and 6000×g for 3–4 minutes, there is an elongation of the egg, which, if the centrifugation is prolonged for a few minutes, is fragmented into two parts If the centrifugation is interrupted before the egg becomes fragmented, there is a stratification visible in the cytoplasm due to an arrangement of the cytoplasmic components according to their specific gravity

All the substances used in this investigation were found to have distinctive effects on the egg surface and the cytoplasm and they all enter the egg rapidly The concentrations have been varied but in most experiments the concentrations have ranged between 10^{-6} M and 2×10^{-3} M

In the presence of chloramphenicol the eggs are more easily stretched and the stratification is likewise facilitated Nicotine had also immediate effects on the egg There was a very accentuated elongation of the egg which also became clearly stratified Fragmentations occurred already within the course of 4 minutes centrifugation at 4000 × g which indicates an exceptional decrease in the rigidity of the egg Thalidomide had also a clear and immediate decreasing action on the rigidity of the egg As in the experiments with nicotine fragmentations occurred within the course of 4 minutes centrifugation The eggs likewise showed much more stratification in the presence of thalidomide than in the control In chlorpromazine the eggs become very rigid and there is no or almost no elongation when there are already fragmentations in the corresponding simultaneous control There is also an almost total inhibition in the stratification of the cytoplasm, which is in keeping with the statement above that the eggs become rigid



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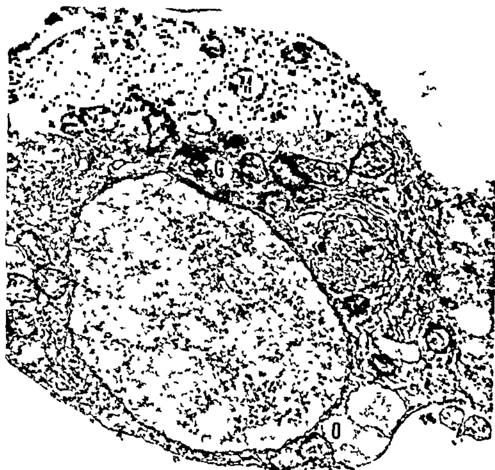


Fig 28 Thalidomide A higher magnification of the syncytium (A) and one of the mesenchyme cells in Fig 27 Note the yolk granules (Y) mitochondria (M) oil droplets (O) and Golgi complex (G) 15 000×

CPZ was found to provoke a partial breakdown of the cortical granules resulting in the formation of membrane blisters These partial membranes are congruent with the fertilization membrane and were formed mainly in the centripetal part of the egg CPZ seems therefore to exert a certain activation of the egg resembling the first steps in fertilization but we never observed any indications of a subsequent nuclear activation or cell division (Figs 29, 30)

The effect of imipramine is similar to that of CPZ though imipramine has a somewhat less pronounced membrane activating action Imipramine also penetrates rapidly into the cell which is demonstrated by an immediate inhibition of the plasma stratification

The results demonstrated that all the substances tested have effects on the surface layers and the cytoplasm of the egg Chloramphenicol, thalidomide and nicotine decidedly decrease the



F g 29 Control egg after centrifugat on for 4 m nutes at 6 000 g.



F g 30 CPZ Centr fugat on as n F g 29 n the presense of 10^{-5} M CPZ No elon gat on of the egg but a membrane bl ster is formed.

displaying the gravest and chloramphenicol the lightest effects at equal molar concentrations

CPZ and imipramine have almost identical effects giving an apparent hard ening of both cell surface and cytoplasm The initiation by CPZ and imi pramine of the cortical reaction is also of considerable interest the more so as there is no general activation of the egg.

Discussion

The study of fertilization and development was initiated by SELENKA (1878) and FOL (1879) about a century ago using the gametes of sea urchins for material. Since then the literature dealing with experiments on sea urchins has grown enormously but still new aspects of developmental biology are first investigated with this material. When working with different organisms the many advantages of the sea urchin egg become apparent. Every female delivers millions of fairly equal transparent eggs which are easily fertilized to 100 per cent and which likewise develop into self-maintaining larvae within the course of a few days. The experimental conditions are easily kept constant and a change, for instance a lowering of the temperature or a variation in the chemical milieu, gives rise to easily detectable differences in the differentiation of the larva.

In the present investigation we have described the effect on fertilization and development of some well-known substances. Besides these substances we have tested more than 40 other substances, mainly clinically used drugs, which will be subject of forthcoming publications.

The substances used here have all been shown to have a strong influence on morphogenesis. In the case of chlorpromazine or imipramine it does not seem likely that the registered effects have very much in common with their pharmacological action in man since no highly organized nervous system is present in the sea urchin larva. This is of course a disadvantage of the material but it is also an asset since it enables us to study the general biological effect of the substance on the cellular level. The results obtained with these two psychopharmaca may, however, also have some bearing on their action in higher organisms. The clear effects seen on the cellular surfaces and the diminished connections between the cells and also the transformations in the cytoplasm and the nucleus probably indicate that there are profound changes in permeability. The same may be true as regards the action of nicotine where also the membranes of the cell are severely affected.

All the substances tested were found to enter the cell rapidly and consequently they act immediately upon the cell as a whole and on the nucleus and cytoplasmic inclusions. Of certain interest is the activating action of CPZ and imipramine and the subsequent formation of partial fertilization membranes. This indicates that proteolytic enzymes are activated since the cortical breakdown is correlated with an increase in these enzymes. It is also possible that this evident rise in a specific enzyme activity may contribute to the reduced cell contacts and the separation of the blastomeres.

It has long been known that CPZ affects the membranes involved in catecholamine transport in nervous tissue and recently it was proposed that CPZ also affects other cellular and subcellular membranes (cf. PALM, GRO-

BECKER & BAK 1970, who also refer to the earlier literature, see also LANGSLET & ØYE 1970) These studies which were carried out on mitochondria of the rat heart and on erythrocytes seem to correspond with our observations of severe effects on subcellular membranes in the sea urchin larva

The separation of the blastomeres after treatments in chlorpromazine is a rather unique phenomenon which we have subjected to a thorough study Cell separation is brought about by treatment even after hatching but is much more easily achieved when the treatment is started during the cleavage stages This fact is probably of importance to the understanding of the underlying mechanism During the development up to hatching there is a very rapid cell multiplication in the embryo After hatching, when the embryo comprises about 800–1000 cells (cf MORGAN 1895) the mitotic activity is greatly reduced since the pluteı consist of less than 3000 cells The consequence is that during the first 10 hours of development the embryo reaches 1000 cells from the fertilized egg cell whereas during the succeeding 40–60 hours the number of cells only increases by a factor of 2–3 During mitosis, and particularly during the interval in which the new cell walls are established, the contacts between the cells are less intimate as compared with the circumstances in the late larva where there are few cell divisions and where the cells are flattened against each other and in contact over an extended part of the cell surface This may afford at least a partial explanation of the difference in response to chlorpromazine of the developmental stages before and after hatching

Though the action of CPZ on cleaving cells and young embryos is certainly not to be compared with the pharmacological affect in man the cell separation exerted by this substance may have some bearing on the physiological action A loosening of the cell contacts may also affect the functioning of nervous tissue At present we do not want to stress this point as our intention is not to produce analogies between the action on the cells of the sea urchin embryo and the action on a highly complex organ system in man

The experiments with thalidomide indicate, on the other hand, that also in sea urchins this substance acts on the mesoderm The skeleton is present though it may suffer some quantitative reductions The primary development of the skeleton appears normal though the final differentiation is characterized by malformations Our experiments with thalidomide on spermatozoa and unfertilized eggs make probable that the substance acts on the genetic material This view is strongly supported by our in vitro experiments with nucleic acids which demonstrated that the teratogenic effect of thalidomide becomes neutralized completely The results with basic amino acids in combination with thalidomide give also new aspects on the toxic action of this drug and may give some reflection on the chemistry of the reaction of thalidomide in the cell

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REFERENCES

Fol, H *Mem Soc Phys Hist nat Genève* 1879, 26, 89
Gibbins, J R. L G Tilney & K R Porter *J Cell Biol* 1969, 41, 201
Gustafson, T & M Toneby *Exp Cell Res* 1970, 62, 102.
Hagstrom, B E. *AABI internal report* 1969, unpublished.
Hagstrom B E. & R. D Allen *Exp Cell Res* 1956 10 14
Hagstrom, B E & B Hagstrom *Exp Cell Res* 1954 6, 479
Hagstrom, B E. & B Hagstrom *Exp Cell Res* 1959, 16 174
Hagstrom, B E. & S Lonning *Arch EntwMech Org* 1967, 158, 1
Hagstrom, B E. & S Lonning *Protoplasma* 1969, 68 271
Harvey E. B *The American Arbacia and other sea urchins*, Princeton 1956.
Hertwig, O & R Hertwig *Jena Z Naturw* 1887, 20 120
Horstadius, S *Devl Biol* 1963 7 144
Kuhn, A *Entwicklungsphysiologie* Berlin 1965
Lallier R. *J Embryol exp Morph* 1962 10 563
Langslet, A & I Öye *Nord Med* 1970 83 553
Lehmann F E. *Einfuhrung in die physiologische Embryologie*, Basel 1945.
Lonning S *Ark Zool* 1959 12 359
Lönning S *Sarsia* 1965 18 17
Lundblad, G *Exp Cell Res* 1950 1 264
Morgan T H *Arch EntwMech Org* 1895 2 81
Okazaki, K *Embryologia* 1960 5 283
Palm, D H Grobecker & I J Bak *Bayer-Symp* 1970 2, 188.
Reynolds, S *J Cell Biol* 1963 17 208
Runnstrom J *Verhandl deut Zool Ges* 1954 p 32.
Runnstrom J *Devl Biol* 1963 7 38
Runnstrom S *Bergens Mus Arbok Naturv Rekke* 1927, No 2.
Selenka, E *Zoologische Studien I Befruchtung des Eies von Toxopneustes variegatus*, Leipzig 1878
Ubisch L. von Z *wiss Zool* 1937 149 402
Ubisch, L von *Entwicklungsprobleme* Jena 1953

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Chloramphenicol was previously used in experiments with sea urchin larvae and fragments of larvae (LALLIER 1962, HORSTADIUS 1963). Our results are not in agreement with those of the previous authors, which might partly be due to differences in the preparations of chloramphenicol used. HORSTADIUS states (loc. cit. p. 145) that "a solution weaker than 0.025 per cent had no effect" and LALLIER (loc. cit.) used still higher concentrations. Our highest concentrations, which had severe effects on differentiation, are well below this concentration. We have tested several other antibiotics and our results with chloramphenicol are in keeping with those obtained from experiments with comparable agents.

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REFERENCES

Fol H *Mem Soc Phys Hist nat Geneve* 1879, 26, 89
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Hagstrom B E *KABI internal report* 1969, unpublished
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VOLUMEN 32, SUPPLEMENTUM II, 1973

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A survey of literature
and own investigations

BY

STEN CHRISTENSEN



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Copenhagen, March 16, 1972

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Answers to these and many other questions require in the first place a more detailed knowledge of the fate of the vitamin in the body The experimental studies preceding this survey were undertaken with the intention of clarifying certain details of the absorption, the distribution, the metabolism and the excretion of the vitamin, and it is the aim of the survey to summarize and discuss the available knowledge within this field Although the experiments were carried out in the rat, the essence of studies carried out in other mammalian species has been included Other biological aspects of riboflavin, e g its physiological functions, pharmacological actions, biosynthesis, antagonists etc, have not been discussed here, but a few reviews on these and related subjects have been listed

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Chapter I

Absorption

The uptake of water soluble vitamins from the intestine shows characteristic differences Some of the vitamins appear to be transported from the intestinal lumen to the blood exclusively by passive diffusion, while others – primarily members of the B group – are known to become absorbed by other mechanisms (MATTEWS 1967) It is of interest that these latter may be characterized by a limited capacity (e g vitamins B_1 and B_{12}) rather than by a high efficiency Active absorption in the sense of transport against an electrochemical gradient has been demonstrated only for biotin (SPENCER & BRODY 1964) and only in certain species

Investigations on the intestinal absorption of riboflavin have been carried out *in vivo* or by means of intestinal preparations Most *in vivo* studies, especially in man, are based on indirect measurements of the absorption rate, frequently by the use of urinary excretion data Such studies provide valid information only in cases where changes in other parameters, e g. renal clearance or rate of metabolism can be excluded *In vitro* techniques for studying absorption processes have been extensively developed over the past 20 years, most of them being applied to riboflavin The advantage of these techniques in providing quantitative data on absorption kinetics is to some extent counterbalanced by the difficulty involved in interpreting these results It is important to realize that *in vitro* preparations of intestine are entirely different from living intestine *in situ*, and that consequently only positive findings can be considered as conclusive

There are several indications that intestinal absorption of riboflavin is species-dependant. Thus, the absorption processes are apparently different in man and in the rat as regards localization as well as efficiency For this reason absorption by man will be discussed separately in the following

Intestinal absorption in man

Rate of absorption. Riboflavin is rapidly absorbed following oral ingestion in humans. Maximum plasma concentration and urinary excretion rate are observed 1–2 hours after the administration (BURCH *et al* 1948, CAMPBELL & MORRISON 1963, STRIPP 1965, LEVY & JUSKO 1966a) An attempt to calculate a first order absorption rate constant from urinary excretion data

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Michaelis-Menten equation. ROY *et al* (1968) reported a maximum excretion of 11 mg following doses up to 100 mg, and MAYERSOHN *et al* (1969) reported 12 mg as the greatest amount that could be excreted following the administration of a single dose.

There is thus considerable evidence that riboflavin, like thiamine and cyobemine (WISEMAN 1964), is absorbed by a saturable mechanism. It is notable that, while the absorption capacity for cyobemine is approximately 2 μg/day, or slightly greater than the requirement (MATTEWS 1967), the capacity for absorption of single doses of riboflavin or thiamine approximates fivefold the daily requirement. Furthermore, as shown by STRIPP (1965), the 'blockade' of riboflavin absorption lasts for only a few hours following one saturation dose. The absorption capacity for riboflavin must therefore be considered as being more than adequate to secure utilization of dietary vitamin.

Site of absorption. The rapid occurrence and short duration of peak levels in the plasma and urine after saturation doses point to the upper intestine as the principal site of absorption. This view is supported by the experiments of CAMPBELL & MORRISON (1963) which indicate that, judged from the urinary excretion data, prolonged availability cannot be obtained by "sustained release" preparations. These authors also demonstrated that preparations with *in vitro* disintegration times of more than 60 minutes resulted in decreased urinary recovery of the vitamin.

Investigations on the absorption from the large intestine are somewhat controversial. NAJJAR *et al* (1944) reported a 'prompt rise in urinary excretion of riboflavin' following administration of 20 mg as a retention enema. EVERSON *et al* (1948), on the other hand, found no extra excretion after rectal administration of 2 mg of the vitamin. Later investigators (CAMPBELL & MORRISON 1963, LEVY & JUSKO 1966a) reported the appearance of 3–6 % of rectally administered riboflavin in the urine. From such data it has been assumed that absorption of the vitamin from the large intestine is normally insignificant (CAMPBELL & MORRISON 1963, LEVY & JUSKO 1966a). However, the mainly negative data might be due to differences between the experimental conditions and the normal conditions for intestinal absorption. The fact that most of the excreted vitamin appears in the urine within the first few hours after administration indicates in itself that absorption occurs mainly in the small intestine. However, regardless of other considerations, the above experiments indicate that riboflavin can be absorbed from the large intestine, and it seems reasonable to suggest that such absorption actually does occur, at least to some extent. Absorption of the vitamin from the large intestine might possibly explain the "slow elimination components" apparent from the urinary excretion data 4–8 hours after oral administration (LEVY & JUSKO 1966a, MAYERSOHN *et al* 1969).

(WIEGAND *et al* 1963) gave values greater than 30 hr^{-1}, indicating a 95 % absorption in less than 6 minutes Although, as pointed out by the authors, rate constants of this magnitude are not meaningful in the applied system, the data do reflect very rapid absorption of the vitamin There are at least two reasons for suggesting that mechanisms other than passive diffusion are involved in the rapid absorption in man Firstly, from physico-chemical considerations the riboflavin molecule would not be expected to penetrate biological membranes so rapidly The molecular weight is relatively high (374), and the chloroform water partition coefficient less than 0 001 (WILSON 1962) Secondly, *in vitro* transport studies have indicated a very low permeability of the intestinal wall to riboflavin (SPENCER & ZAMCHECK 1961, TURNER & HUGHES 1962)

Efficiency and saturation of absorption. Urinary excretion of riboflavin following oral ingestion has been the subject of numerous investigations Some of them were carried out with the purpose of developing diagnostic excretion tests In a different type of studies riboflavin was used as a model substance for studying intestinal absorption in general It was during the latter kind of experiments that a few years ago riboflavin absorption was discovered to be more complex than had been previously believed (STRIPP 1965, LEVY & JUSKO 1966a)

Ignoring earlier reports based on less reliable methods, there is now fairly good agreement that approximately 50 % of oral doses of less than 20 mg is excreted in the urine (KUNZ 1942, MELNICK *et al* 1945, BREWER *et al* 1946, MORRISON & CAMPBELL 1960, STRIPP 1965, LEVY & JUSKO 1966a) While several investigators reported a constant fraction of the dose excreted in the urine, BREWER *et al* observed a greater retention of the vitamin in doses of less than 1 5 mg The proportional relationship between the dose and amount excreted supported the widespread view that riboflavin, as opposed to vitamins B_1 and B_{12}, is absorbed by passive diffusion (MORRISON & CAMPBELL 1960, WISEMAN 1964) However, recent studies with greater doses of vitamin (STRIPP 1965, LEVY & JUSKO 1966a, JUSKO & LEVY 1967a, RYU *et al* 1968, MAYERSOHN *et al* 1969), as well as a previous study by LANE *et al* (1958), indicate that orally administered riboflavin can only be excreted in amounts up to a certain limit In this laboratory STRIPP (1965) demonstrated that the total excretion cannot exceed 14–18 mg, not even if the dose is increased to 500 mg of the vitamin She also noticed that the peak levels of riboflavin in the plasma were the same whether 50, 100, or 500 mg doses had been given to the same subject. This observation indicates that the limited urinary excretion is due to a limited absorption from the intestine Independently, LEVY & JUSKO (1966a) found a similar limitation of the amount of vitamin excreted in the urine They further showed that the relationship between administered and excreted amounts applies to the

Intestinal absorption in animals.

Intestinal absorption of riboflavin has been studied in animals by more direct techniques than can be used in man. Investigations may be divided into *in vivo* experiments dealing with the disappearance of riboflavin from the intestine following oral administration or injection into ligated loops of intestine, and *in vitro* experiments, showing the ability of various intestinal preparations to transport or accumulate the vitamin. Most studies have been carried out in the rat, this animal being used if nothing else is indicated.

Kinetics and efficiency of absorption. The fraction of orally administered riboflavin appearing in the urine is in general lower in animals than in man. Thus in the rat 5–10 % of oral doses up to 2 mg is excreted unchanged in the urine (SURE & FORD 1943, CHRISTENSEN 1969a). Since the amount excreted in the faeces is only 10–20 % of the dose (CHRISTENSEN 1969a) the low urinary recovery may not be due to limited absorption, but rather to elimination by other routes (e.g. degradation). Alternatively, the low faecal output may be attributed to destruction of the vitamin within the intestinal tract, a possibility which will be discussed further in chapter 3. Since urinary excretion accounts for a minor part of the elimination, it may not be valid to indicate the extent of the intestinal absorption. If, however, the absorption capacity could be saturated by high doses as is the case in man the percentage of the dose excreted in the urine, although small, might be expected to decrease. Since this is not observed even after high doses (CHRISTENSEN 1969a) urinary excretion data argue against a saturable absorption process.

A more direct approach to a study of the absorption kinetics was attempted by the following (unpublished) experiments on the rat. During halothane anaesthesia the abdomen was opened and 1 ml isotonic solution of riboflavin or FMN injected into 10 cm ligated loops of small intestine. One hour after the injection the animals were decapitated and the above mentioned segments removed, rinsed with water, stripped, weighed and frozen. Rinsing fluid and homogenized tissue were analyzed for riboflavin, FMN and FAD by the method of STRIPP (1965) and the amount absorbed determined as the amount injected less the amount in the rinsing fluid. Fig. 1 indicates the absorption of riboflavin from proximal (duodenum + part of *jejunum*) and distal (ileum) segments of the small intestine. In both segments the amount removed from the intestinal lumen increased proportionally to the amount injected, but the percentage disappearance was greater from proximal than from distal segments. In additional experiments with FMN the amount of flavin absorbed from each segment was increased arbitrarily with the amount injected up to 500 μg/g intestine. Thus, the experiments confirmed the concept of no upper limit for the absorption. FMN was rapidly dephos-

Factors influencing the absorption. JUSKO & LEVY (1967a) *reported that* the amount of riboflavin excreted in the urine increased providing the vitamin was administered immediately after a meal This apparent *effect of food* on the absorption was attributed to delayed gastric emptying associated with prolonged retention of the vitamin at the sites of absorption Bile salts have been shown to enhance absorption in man (MAYERSOHN *et al* 1969) and in the dog (ONISHI 1956), and to increase the *in vitro* permeability of the rat small intestine (MAYERSOHN *et al* 1969) These findings are consistent with the recent observation that riboflavin absorption is impaired in children with biliary obstruction (JUSKO *et al* 1971)

An inhibitory effect of probenecid on the absorption was suggested by JUSKO & LEVY (1967b), since this compound decreased the *early* urinary excretion rate of orally administered riboflavin It is now well established, however, that probenecid blocks tubular secretion of riboflavin (JUSKO *et al* 1970a), and this in itself might explain the effect on urinary excretion

Mechanism of absorption. The following characteristics of riboflavin absorption should be considered (i) The rate of absorption may exceed that expected for passive diffusion, (ii) the absorption is saturable, (iii) the absorption exhibits site specificity, and (iv) the efficiency of absorption is enhanced by food or bile salts All these characteristics together support the view that, in man, riboflavin absorption is mediated by a "specialized" transport process rather than by passive diffusion The additional nature of this process (active transport, facilitated diffusion, etc), as well as its molecular basis, are unknown It has been suggested that phosphorylation dephosphorylation processes may be involved (JUSKO 1970) However, since evidence of intestinal phosphorylation is limited to animals (cf the following section), and since an effect of probenecid (which may inhibit phosphorylations) on the absorption can be questioned, there is little basis for this *hypothesis*

The reason why bile salts enhance the absorption is uncertain MAYERSOHN *et al* (1969) point out that bile salts may affect proximal intestinal transit or membrane permeability, or that they may solubilize the vitamin in the intestinal lumen The possibility can be excluded that the solubility of the vitamin (approximately 50 mg/litre in water) is a limiting factor, since riboflavin-5′-phosphate (FMN), a compound readily soluble in water, is absorbed from the intestine with the same efficiency and saturation threshold as riboflavin (JUSKO & LEVY 1967a) Although FMN is known to be rapidly dephosphorylated to free riboflavin in the small intestine (OKUDA 1958a, b, CHRISTENSEN 1969b) and is apparently absorbed in this form, it is unlikely that riboflavin originating from intestinal dephosphorylation of FMN should precipitate in the gut at the actual doses

hour, i ' ... during the concentration gradient from intestinal fluid to blood

in vitro ...

to riboflavin (table 1) No more than two thirds of the riboflavin injected into ligated loops of intestine has been found to disappear from the lumen in 1-21 hours. Everted sac experiments, carried out by the technique of WILSON & WISEMAN (1954) indicate that an equilibrium between mucosal and serosal fluids is far from being reached within one hour In the studies referred to in table 1, the transport from mucosal to serosal fluid was of the same order of magnitude as the transport in the opposite direction Neither experiments with everted sacs, nor experiments with isolated mucosal cells (GOLMAN 1970) have revealed transport against a concentration gradient It should be noted that thiamine, another B vitamin known to be absorbed by a saturable mechanism in man, has been shown to be actively transported into rat mucosal cells (MENON & SUGNEN 1971), but not into gut sacs (TURNER & HUGHES 1962)

Site of absorption MIDDLETON & GRICE (1964) determined the amount of radioactivity in different parts of the intestinal tract after oral administration of tracer doses of riboflavin ^{14}C Maximum disappearance rate of radioactivity occurred one to four hours after administration associated with its

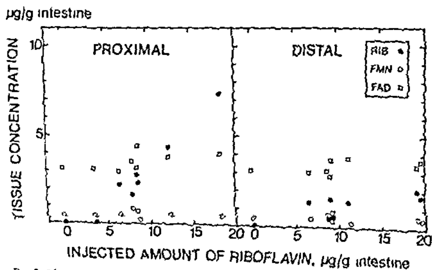


Fig 2 Flavin concentrations in the intestinal wall of proximal and distal segments of rat small intestine as influenced by intraluminal injections of riboflavin. The segments were removed 1 hour after the injections and rinsed with water before analysis (same experiments as in fig 1).

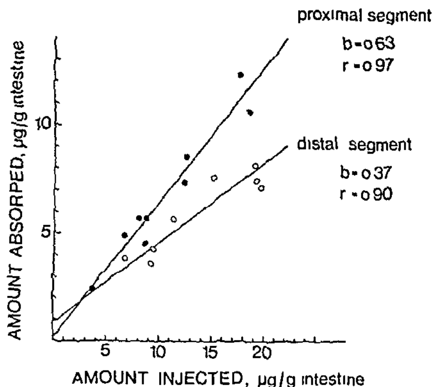


Fig 1 Absorption of riboflavin *in vivo* from ligated loops of rat small intestine The abcissa indicate the amounts of riboflavin injected into proximal or distal segments of the small intestine The ordinate indicate the amounts which disappeared from the lumen within 1 hour The straight lines have been drawn according to the least squares principle b = the slope, r = the correlation coefficient

phorylated to free riboflavin in the intestinal lumen. The extent of dephosphorylation in the course of one hour was 97–100 % in the proximal segments and 80–90 % in the distal segments This is in agreement with the finding in dogs (OKUDA 1959) that mucosal dephosphorylation activity is greater in the duodenum and the jejunum than in the ileum

Fig 2 indicates the content of riboflavin and its coenzyme derivatives in the intestinal wall as influenced by riboflavin injections into ligated loops Only free riboflavin is increased in the tissue, and the increase is dose-dependent and considerably greater in proximal than in distal segments This tendency of riboflavin to accumulate in the wall of the proximal small intestine was even more pronounced after large doses of FMN Thus, following injection of 300 µg/g intestine, no less than 204 µg/g was found in the proximal intestinal wall as opposed to 12 µg/g in the distal wall Likewise, only the amount of free riboflavin was increased in the tissue on injections of FMN

The rate of absorption estimated from these experiments, 40–63 % in one

[illegible] the concentration gradient from intestinal fluid to [illegible] and [illegible]

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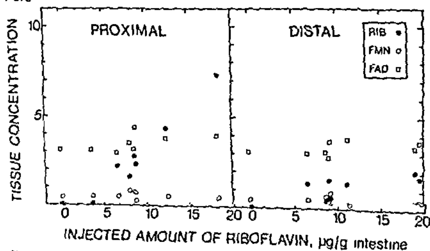


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Table 1

Transport of riboflavin by rat small intestine *in vivo* or *in vitro*

Technique	Duration of experiment (hours)	Amount injected (µg) / Initial mucosal conc (µg/ml)	Amount removed (%) / Mucosal/serosal conc ratio	Authority
Ligated loops	2½	125 µg	64 %	LASZT & DALLA TORRE 1943
Ligated loops	2½	125 µg	59 %	PONZ 1944
Ligated loops	1	375 µg	34 %	SPENCER & ZAMCHECK 1961
Ligated loops	1	10–400 µg	40–63 %	CHRISTENSEN (unpublished)
Everted sacs	1	150 µg/ml	3	SPENCER & ZAMCHECK 1961
Everted sacs	1	8 23 µg/ml	3–10	TURNER & HUGHES 1962
Isolated mucosal cells	1–3	1 µg/ml	no accumulation	GOLMAN 1970

localization in the lower ileum and caecum Further experiments indicated that, while up to 70 cm of the ileum could be removed without a decrease in the radioactivity excreted with the urine, caecectomy caused a significant decrease in the excreted radioactivity

Since riboflavin is known to be extensively metabolized following oral ingestion in rats (CHRISTENSEN 1969b, 1971b), radioactive data may not indicate migration of unchanged vitamin within the body Exchange with endogenous riboflavin may be a further source of failure, especially following small radioactive doses For these reasons the above experiments were repeated using larger doses and determining riboflavin fluorimetrically One mg of FMN dissolved in 2 ml of water was administered by stomach tube to female Wistar rats weighing 200 g without access to food 24 hours before and during the experiment Animals were decapitated at various times after the administration, and the entire gastrointestinal tract removed, ligated and divided into segments which were analyzed for total riboflavin The recovery of riboflavin in each segment was estimated by subtracting the flavin contents of segments from animals which had not received riboflavin Fig 3 shows the recovery in each segment at various intervals of time It is clear that the main absorption occurs later than two hours after the administration at a point when the vitamin is located mainly in the lower ileum and the caecum This is in agreement with the results of MIDDLETON & GRICE (1964) It is further characteristic that the absorption occurs over a wide period as

opposed to the prompt absorption in man This prolonged absorption, which is apparently due to slow absorption throughout the intestine, explains the lack of a definite peak in the plasma riboflavin level In the actual experiment the plasma riboflavin was almost the same from 2 to 24 hours after the administration At the same time the maximum increase in plasma riboflavin (0 02 µg/ml or 40 %) was much lower than that seen in man following oral saturation doses (0 25 µg/ml or 500 %, STRIPP 1965)

Absorption from the large intestine in the rat has been demonstrated by the injection of riboflavin directly into the caecum. MIDDLETON & GRICE *(1964) reported that radioactive vitamin administered in this way was at least* 75 % as 'available as the vitamin administered orally KASPER (1965) used the weight gain of riboflavin depleted animals as a measure of absorption, and showed that riboflavin was utilized equally well whether administered orally or injected into the caecum Histochemical examinations of rat intestinal tissue following oral doses of riboflavin have shown that the vitamin penetrates into the mucosa of the small and large intestine, but scarcely into that of the stomach (CHEN & YAMAUCHI 1961)

In what form is riboflavin absorbed? The absorption process may be di-

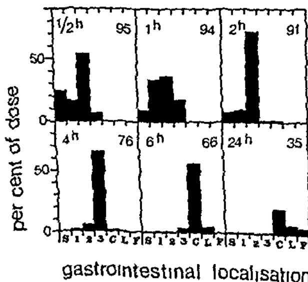


Fig 1 Recovery of total riboflavin from various parts of the gastro-intestinal tract of the rat at different times after oral administration of 1 mg of FMN Figures in upper left corners indicate hours after the administration. Figures in upper right corners indicate the percentage recoveries from the whole gastro-intestinal tract Key for the abcissa S = stomach 1 = proximal, 2 = medial 3 = distal segments of small intestine C = caecum L = large intestine and F = faeces.

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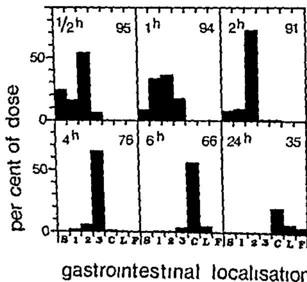


Fig 3 Recovery of total riboflavin from various parts of the gastro-intestinal tract of the rat at different times after oral administration of 1 mg of FMN Figures in upper left corners indicate hours after the administration Figures in upper right corners indicate the percentage recoveries from the whole gastro-intestinal tract Key for the abcissa S = stomach 1 = proximal 2 = medial, 3 = distal segments of small intestine, C = caecum L = large intestine and F = faeces

vided into two steps Transport from the intestinal lumen into the mucosal cells, and transport from these cells via the interstitium into the blood capillaries The first step is undoubtedly performed by unchanged riboflavin since phosphorylation is unlikely to occur within the intestinal lumen On the contrary, phosphorylated vitamin will, as shown in the ligated loop experiments, be rapidly dephosphorylated in intestinal juice There are many indications, however, that riboflavin is at least in part phosphorylated within the mucosal cells, and that phosphorylated vitamin (FMN) is transported into the blood stream In dogs, KAGAYA (1956) demonstrated increased concentrations of riboflavin as well as of FMN in the portal blood after oral administration of riboflavin ONISHI (1956) detected riboflavin as well as FMN in the serosal fluid of perfused dog intestine when riboflavin was added to the mucosal solution MUTO (1959) reported that more than half of the flavin appearing in the serosal fluid of isolated rat intestine, was FMN if only riboflavin was present in the mucosal fluid CHEN & YAMAUCHI (1960 1961) made histochemical investigations on the absorption from rat intestine *in vitro* and *in vivo*, and observed that part of the riboflavin became phosphorylated while passing through the small intestinal mucosal cells They further reported that FMN and riboflavin are equally well transported through the mucosa, while FAD is but poorly transported However, they presented no quantitative data MIYAO *et al* (1967) reported that approximately one third of the flavin transferred to the serosal solution by surviving rat intestine was FMN In the everted sac of chicken intestine CORDONA & PAYNE (1967) found that, following addition of riboflavin to the mucosal side, two thirds of the flavin in the serosal solution was FMN

In the ligated loop experiments described here, no increase was observed in the amount of FMN in the intestinal wall during absorption of riboflavin or FMN This observation is apparently inconsistent with the numerous reports suggesting phosphorylation of the vitamin during its absorption Phosphorylation might, however, possibly take place without any significant accumulation of phosphorylated vitamin in the tissue if the FMN is rapidly removed by the perfusing blood Except for the histochemical study *in vivo* by CHEN & YAMAUCHI (1961), evidence of intestinal accumulation of FMN has been obtained exclusively in intestinal preparations without blood perfusion

The ability of the intestinal mucosa to phosphorylate riboflavin has been known for a long time, but its relation to the absorption process remains uncertain RUDY (1935) and PULVER & VERZAR (1939) reported phosphorylation of riboflavin by extracts of rat small intestine to which inorganic phosphate was added i e apparently by a 'reverse phosphatase' reaction However, their results could not be reproduced by later investigators (KEARNEY & ENGLARD 1951) and only by using various organic phosphate donors did

YAGI & OKUDA (*1958*) *succeed in* phosphorylating riboflavin by means of a highly purified phosphomonoesterase preparation of dog mucosa. ENGLARD (*1952*) *demonstrated* phosphorylation by a flavokinase reaction *in preparations of rat intestine* with ATP added as a phosphate donor In view of the considerable phosphatase activity normally present in preparations of intestine (OKUDA 1958*b*, 1959), *phosphorylation of riboflavin in vivo is presumably* due to flavokinase reactions, rather than to "reverse phosphatase" reactions

The fact that mucosal cells contain enzymes causing both phosphorylation and dephosphorylation of riboflavin, does of course give rise to speculation about the absorption mechanism. At present, however, there is no evidence that phosphorylation is necessary for the absorption. YOSHIHARA (*1954*) reported that, in rabbits, there is no correlation between the rate of absorption and *phosphorylation in different parts of the intestine*. It is further uncertain whether the phosphorylation actually occurring within the mucosal cells during the passage of vitamin will promote its absorption It should be noted that parenterally administered riboflavin is also phosphorylated in the small intestinal mucosa, and it has been suggested that this tissue plays an important role in the overall phosphorylation of the vitamin (YAGI 1954a)

Factors affecting the absorption. LASZT & DALLA TORRE (1943) reported that the amount of riboflavin absorbed from ligated loops of rat intestine was decreased in adrenalectomized animals This impaired absorption was attributed to the lack of adrenocortical hormones believed to control intestinal phosphorylation or permeability However, the profound changes in bodily processes following adrenalectomy necessitate alternative interpretations Using a similar technique, PONZ (1944) demonstrated *impairment of riboflavin* absorption due to the administration of thyroid hormone, which was believed to inhibit phosphorylation. This hypothesis is inconsistent with the present knowledge, according to which thyroid hormone stimulates the activity of flavokinases, i e the enzymes which catalyzes phosphorylation of the vitamin (RIVLIN 1970b) *In fact, because of the low tissue concentration of* free riboflavin observed in hypothyroidism it has been suggested that thyroid hormone does actually promote the absorption from the intestine (RIVLIN 1970b)

A cation effect on riboflavin transfer by the everted rat intestine was recently described by MAYERSOHN & GIBALDI (1970) Replacement of sodium in a Krebs-bicarbonate buffer by potassium, ammonium or guanidinium reduced the mucosa serosa transfer of riboflavin by up to 50 % *Since* cation replacement also interfered with the transfer of salicylate and sulphanilamide, the decreased permeability to riboflavin by cations cannot be attributed to *impairment of any specific* absorption mechanism

An increasing effect of bile salts on riboflavin absorption in the dog and in the rat was mentioned on page 11

Mechanism of absorption. On the whole, available data suggest that riboflavin in the rat, and probably in other animals, is absorbed from the intestine by passive diffusion

The absorption rates determined *in vivo* or *in vitro* are slow, as is to be expected considering the physico chemical properties of the molecule On the other hand, the transport rates determined *in vitro* have been calculated to be sufficient for securing utilization of riboflavin present in usual amounts in the diet (SPENCER & ZAMCHEK 1961, TURNER & HUGHES 1962) There are no indications that the transepithelial transport can be saturated at high vitamin concentrations "Uphill" transport has not been demonstrated, and inhibitors of active transport do not influence riboflavin transference by the intestine *in vitro* (TURNER & HUGHES 1962) Of course, for methodological reasons, these negative *in vitro* findings are not in themselves conclusive, but, unlike the situation in man, there are no *in vivo* data suggesting a specialized absorption mechanism

Data indicating preferential absorption from the lower ileum and caecum do not provide evidence against passive diffusion, since the transit of intestinal contents is known to decrease exponentially along the small intestine of rats (SCHOTTEK 1966) The fact that more vitamin disappears from the proximal than from the distal loops of the small intestine may be due to the relatively greater surface of the proximal mucosa (WOOD 1944)

The data indicating specific accumulation of riboflavin in the proximal intestinal wall are not immediately compatible with a passive diffusion mechanism However, this accumulation may not necessarily be related to the absorption of the vitamin, but may reflect a phosphorylation (YAGI 1954a) or mucosal secretion mechanism located in the upper part of the small intestine According to the latter possibility, evidence for intestinal secretion of the vitamin is presented in chapter 4 The relationship between phosphorylation and absorption of the vitamin by the intestine is not yet clear Part of the absorbed riboflavin appears in the portal blood in a phosphorylated form, but there is no indication of this phosphorylation being necessary for its transfer

Utilization of riboflavin produced by the intestinal microflora.

Unlike higher animals, several microorganisms are able to synthesize isoalloxazines, and riboflavin produced by the microflora in the gastro-intestinal tract may be utilized by the host organism to a varying extent This symbiotic relationship is well known in ruminants, which are independent of exogenous sources of essential amino acids and vitamins B because of their characteristic ruminal microflora (cf OWEN & WEST 1970) In non ruminants, the extent to which the requirement can be provided by intestinal biosynthesis

varies from species to species, and within a single species it depends on the composition of the diet. The fact that symbiotic vitamin can be utilized in non ruminants even if coprophagy is prevented, provides additional evidence for the absorption of the vitamin from the large intestine.

The influence of the diet is well exemplified in the rat. Young animals fed on a riboflavin free synthetic diet with sucrose as the only carbohydrate will soon stop growing. However, when sucrose is replaced by less digestible carbohydrates such as starch, sorbitol or lactose, the animals will grow almost as rapidly as if riboflavin had been supplied (FRIDERICIA *et al.* 1927, HAENEL *et al.* 1959). Further, the excretion of riboflavin in the urine or faeces will increase if sucrose in the diet is replaced by lactose or starch, while it will decrease if sulpha drugs are included in the diet (DE & ROY 1951). Hence, the influence of the diet on the utilization of "symbiotic" vitamin seems to depend on its suitability as a growth medium for riboflavin producing strains of microorganisms.

It has been suggested that under certain conditions also humans may utilize riboflavin produced by the intestinal microflora. NAJJAR *et al.* (1944) demonstrated that the total amount of vitamin excreted in the urine and faeces over a long period with a very low intake might exceed the amount ingested by 5–6 fold without any development of deficiency. Since by far most of the vitamin was excreted with the faeces, the extent of utilization cannot be calculated from the data, but they indicate nevertheless the ability of the human microflora to synthesize riboflavin. RAMA SASTRI *et al.* (1950) reported that on a high-carbohydrate and a low fat diet, human subjects excreted 40–250 % of the ingested vitamin in the urine, and that the excretion was related to the ratio of carbohydrate to fat in the diet rather than to the intake.

The varying ability of the mammalian species to utilize riboflavin produced by intestinal microorganisms may be related to differences in the diet, in microflora, and probably in their ability to absorb the vitamin from the large intestine. The presence of vitamin in the large intestine is by no means tantamount to its availability for the host. For instance, LUCKEY *et al.* (1955) observed that chickens dying of riboflavin deficiency contained sufficient riboflavin in their caecum and rectum to save their lives provided it was administered orally.

Absorption from parenteral sites.

The absorption of riboflavin following parenteral routes of administration has not been systematically investigated but parenteral administration has been widely used for experimental purposes. Riboflavin – injected as a solution – seems to become more rapidly and completely absorbed from the

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Chapter 2

Distribution

Riboflavin, and particularly its coenzyme forms flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are essential constituents of all living cells and hence widely distributed in the body It may be convenient, therefore, to distinguish between two aspects of riboflavin distribution The 'basal' distribution, i e. the contents of endogenous flavins in the tissues as influenced by the dietary supply, and the more short term "dynamic" distribution pattern of excessive *vitamin administered as single doses* The present *survey deals preferentially with the dynamic* behaviour of administered vitamin, but for obvious reasons the basal distribution must be considered also *The binding of flavins to proteins and the transport* of flavins across biological membranes are additional subjects treated in this chapter

Distribution of endogenous flavins

The flavin content of mammalian tissues has first and foremost been studied in the rat Table 2 lists the concentrations of riboflavin, FMN and FAD in various rat tissues, arranged in the order of decreasing total flavin content The data have been collected from four separate studies, but they may be considered comparable, since the flavin content of tissues included in more than one study is in good agreement The greatest content is found in the kidney the liver and the heart, but also the gastro-intestinal tract and various glands are rich in flavins Brain, striated muscle and skin contain much less flavin The flavin content in blood cells and plasma is extremely low as compared with the content in tissues As a rule, FAD – the predominant form of the vitamin – constitutes 80 % or more of the total content. FMN accounts for most of the remaining flavin, concentrations of free riboflavin being almost negligible Exceptions from this pattern are seen in the kidney which contains relatively much FMN, and in the blood plasma, where free riboflavin accounts for approximately half of the total flavin There is an obvious correlation between the flavin content and the metabolic activity of the tissues This is not surprising considering the importance of flavin coenzymes in energy metabolism

YAMAGIRI (1958) studied the *intracellular* distribution of flavin in rat liver by means of a differential centrifugation technique Of the total flavin

subcutis, the striated muscles and the peritoneal cavity than from the gastrointestinal tract In man, JUSKO & LEVY (1967b) reported a rapidly onsetting and sometimes complete (60–100 %) urinary excretion of single intramuscular doses of FMN In the rat, intraperitoneal or subcutaneous injections of dissolved riboflavin result in 24-hour urinary recoveries of around 50 % (CHRISTENSEN 1971b), i e considerably more than after oral administration Absorption from the subcutis is delayed if riboflavin is injected as a suspension, only 5–10 % being excreted in the urine within the first 24 hours (CHRISTENSEN 1971b)

Riboflavin suspensions with addition of 2 % aluminium monostearate have been suggested for depot injection therapy (BRZEZINSKI *et al* 1957) In patients, one 50–150 mg dose of this preparation injected intramuscularly produced increased urinary levels of the vitamin for 4–6 weeks

diluted to the lower concentration limit, after which the body weight will diminish to compensate for the inevitable loss of riboflavin by degradation and excretion During deficiency, all three flavins decrease in the tissues, but as a rule a greater proportion of FMN than FAD is lost. Maximum growth stimulating effect is obtained with supplies producing approximately 75 % saturation of the tissues

The characteristic upper and lower limits in tissue flavin content have not been satisfactorily explained. A likely hypothesis as regards the upper limit is that the flavins attached to the tissue flavoproteins are much less susceptible to elimination processes such as hydrolysis, excretion and degradation, than are unbound flavins (BRO-RASMUSSEN 1958, RIVLIN 1970b) Thus, the amount of flavoproteins available in each tissue might be an upper limiting factor This explanation gains support from the fact that the critical point in the urinary excretion (i e. the dose level above which a greater proportion of the vitamin is excreted) approximates the dose level that produces tissue *saturation in the rat* (BRO-RASMUSSEN 1958)

Distribution of administered riboflavin.

Animals supplied with sufficient amounts of riboflavin usually contain maximum flavin levels in their tissues For this reason, administered riboflavin cannot be retained in the body, but is exposed to rapid elimination processes The initial plasma half life of administered riboflavin is approximately 12 minutes in the rat (CHRISTENSEN 1969c) and 80 minutes in man (calculated from data by STRIPP 1965)

Following parenteral administration of riboflavin to rats, a significant rise occurs momentarily in the flavin content of the kidney, the liver and the intestine, whereas the flavin content of other tissues such as the brain, muscle or skin remains unchanged or exhibits only a slight increase (fig. 4) (YAGI 1954a, YAGI *et al* 1966) The intestine exhibits the greatest increase, resulting from riboflavin as well as from FMN, whereas in the liver and in the kidney, the FAD content increases almost selectively This "acute" pattern of distribution has given rise to the suggestion that the intestine may be an important site for phosphorylation of the vitamin, and that the kidney and liver are important tissues in the FAD biosynthesis (YAGI 1954a) This interpretation has gained support from studies with radioactive riboflavin (YAGI *et al* 1966) and from investigations on the synthesis of flavin coenzymes by tissue preparations (ENGLARD 1952, MCCORMICK 1961) These tissues are, however, not unique in the synthesis of flavin coenzymes Preparations of brain, spleen and heart tissue also exhibit considerable flavokinase activity (MCCORMICK 1961), and even blood cells have been shown to synthesize FAD from added riboflavin *in vitro* (KLEIN & KOHN 1940)

Table 2

Distribution of endogenous flavins in the tissues of rats fed with optimum amounts of riboflavin. The data have been collected from YAGI 1954a, BESSEY *et al.* 1949 (*), BURCH *et al.* 1948 (**) and BESSEY *et al.* 1958 (***)

Tissue	μg per g wet tissue			
	Total	FAD	FMN	RIB
Liver*	40.2	33.2	6.2	0.8
Kidney*	40.1	27.3	11.9	0.9
Adrenals*	22.3	18.3		
Heart	20.5	19.7	0.8	0.1
Stomach	7.70	6.78	0.78	0.14
Intestine	7.65	5.60	1.86	0.19
Spleen	6.72	6.05	0.54	0.13
Submaxillary gland*	5.77	4.65		
Lung*	4.27	3.64		
Testes	3.81	2.70	1.02	0.09
Whole body***	3.8			
Brain	3.41	2.74	0.61	0.06
Striated muscle	3.01	2.75	0.22	0.04
Skin*	1.00	0.76		
Blood cells**	0.14			
Blood plasma**	0.032	0.014	0.002	0.016

content in the liver, 18 % was found in the nuclear, 65 % in the mitochondrial, 4 % in the microsomal and 14 % in the soluble fraction. FAD accounted for 81 % of the total tissue flavin, while the relative FAD content of individual fractions varied between 95 % in the mitochondria and 64 % in the cytoplasma. The remaining flavin largely originated from FMN, free riboflavin being present only in negligible amounts.

The relationship between dietary supply of riboflavin and tissue flavin concentrations has been extensively studied in the rat by Bessey, Burch and their collaborators (BURCH *et al.* 1948, BESSEY *et al.* 1949, BURCH *et al.* 1956, BESSEY *et al.* 1958). Their results may be summarized as follows. For each tissue there is an upper limit for the flavin content which cannot be exceeded, no matter how much vitamin is fed. Further, there is a lower limit below which the content will not fall on a riboflavin-free diet, no matter how long continued. The characteristic upper and lower limits differ as little as 35 % in certain tissues, e.g. in the brain, whereas in the liver – at the other extreme – there is a 4-fold difference between "ceiling" and "floor" levels. Riboflavin supplied in excess of the amount giving rise to maximum tissue concentrations is not retained. On the other hand, if an animal is placed on a riboflavin free diet, it will grow until its tissue flavin has been

diluted to the lower concentration limit, after which the body weight will diminish to compensate for the inevitable loss of riboflavin by degradation and excretion. During deficiency, all three flavins decrease in the tissues, but as a rule a greater proportion of FMN than FAD is lost. Maximum growth stimulating effect is obtained with supplies producing approximately 75 % saturation of the tissues.

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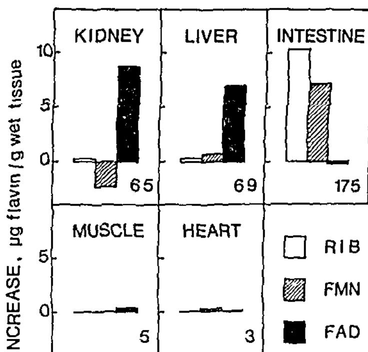


Fig 4 Changes in the flavin content of various rat tissues recorded 1 hour after injection of 300 µg of riboflavin subcutaneously Figures in lower right corners indicate increases of the total flavin content Calculated from data of YAGI (1954a)

These data, as well as the wide distribution of enzymes mediating the synthesis of FMN and FAD (see chapter 3), suggest that most tissues are able to produce their 'own" flavin coenzymes It is probable, therefore, that free riboflavin serves as the predominant transport form, which is taken up by the individual tissues from the blood plasma, and thereafter converted to its coenzyme forms

A considerable proportion of parenterally administered riboflavin accumulates in the intestine YAGI (1954a) reported the vitamin localized mainly in the mucosa, but did not determine the amount of flavin in the intestinal contents The fact that parenterally administered riboflavin is to a great extent excreted in the faeces (CHRISTENSEN 1971b) indicates secretion of the vitamin from the blood into the intestinal lumen In order to elucidate this problem and further to obtain information as regards the regional distribution of intestinal secretion, the following experiment was made

In an anaesthetized female Wistar rat the entire *gastro intestinal tract* was ligated into segments, and riboflavin ~ 0 5 mg/kg/hour ~ was infused intravenously for 2 hours The animal was then decapitated and each segment removed, and the contents collected by washing with water Total riboflavin was determined in the tissue and rinsing fluid, and the amount

each segment calculated (table 3). Fourty-five per cent of the dose was recovered in the entire gastro-intestinal tract, which represented approximately 7% of the body weight. Most riboflavin was found in the proximal and medial parts of the small intestine, less in the distal small and large intestine, and practically none in the stomach. Taken as a whole, equal amounts were found in the intestinal tissue and in the contents of the intestinal tract, but whereas most of the flavin was bound to the tissue in the small intestine, the opposite was the case in the large intestine.

These results indicate that riboflavin accumulates in the intestinal wall, presumably in the mucosa, from which it is secreted into the intestinal lumen. The relatively low secretion into the duodenal segment suggests that biliar secretion is not predominant in the transport of the vitamin into the intestine. However, in an experiment with a five fold higher infusion rate, the greatest amount was found in the duodenum, suggesting that biliary secretion may play a significant role at higher plasma concentrations. This is in accordance with recent studies on the biliary excretion of the vitamin (NOGAMI *et al.* 1970), as discussed further in chapter 4.

Apparent volume of distribution.

In the rat, the specific accumulation of administered riboflavin in certain tissues causes a relatively slight increase in the flavin content of other tissues and of blood plasma (YAGI 1954a, CHRISTENSEN 1969c). Accordingly, estimates of the apparent volume of distribution may considerably exceed the

Table 3

Recovery of total riboflavin in tissue and contents of various parts of the gastro-intestinal tract following 2 hours slow intravenous infusion of riboflavin to an anaesthetized rat. Body weight 155 g. Total dose 155 µg

Part of the gastro-intestinal tract	Wet weight of tissue g	Per cent of the dose recovered in		
		tissue	contents	total
Stomach	1.33	0.0	0.6	0.6
Small intestine 0–20 cm	1.43	2.8	4.6	7.4
Small intestine 20–52 cm	2.01	10.5	4.3	14.8
Small intestine 52–80 cm	1.75	8.0	4.4	12.4
Small intestine 80–96 cm	1.15	2.0	0.0	2.0
Caecum	1.04	0.0	4.2	4.2
Large intestine	1.51	0.4	2.9	3.3
Gastro-intestinal tract	10.22	23.7	21.0	44.7

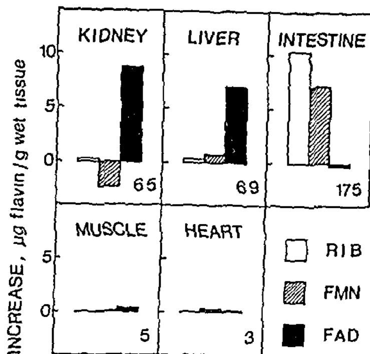


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Small intestine 52–80 cm	1 75	8 0	4 4	12 4
Small intestine 80–96 cm	1 15	2.0	0 0	2 0
Caecum	1 04	0 0	4 2	4.2
Large intestine	1 51	0 4	2.9	3.3
Gastro-intestinal tract	10 22	23 7	21 0	44 7

body weight (CHRISTENSEN 1969c, NOGAMI *et al* 1970) Unpublished estimates based on renal clearance experiments (CHRISTENSEN 1971a) resulted in values ranging from half to fourfold the body weight The difficulty in obtaining reproducible measures of the distribution volume indicates that, in the rat, unrecognized experimental conditions may affect the distribution and/or elimination pattern of the vitamin

More reliable estimates have been obtained in the dog JUSKO *et al* (1970c) reported apparent distribution volumes from 15 to 30 % of the body weight in four animals, as calculated by nonlinear computer fits to constant rate infusion data In man, calculations from apparently monoexponential plasma and urine elimination data (STRIPP 1965) yielded values from 25 to 39 % of the body weight (LEVY & JUSKO 1966b) However, careful pharmacokinetic analysis has indicated that plasma and urine elimination data following single injections of FMN to man and dog are best fitted to triexponential equations (JUSKO & LEVY 1970) On this basis a multiple-compartment open model has been proposed as the most likely, and the volume of the central compartment has been calculated to 16 % of the body weight in both species (JUSKO & LEVY 1970) Since injected FMN is partly dephosphorylated in the blood (CHRISTENSEN 1969c, JUSKO *et al* 1970a), and since only total flavins were determined in the plasma, it is, however, uncertain to what degree this figure applies to free or phosphorylated riboflavin

Regardless of possible sources of failure, the volume of distribution seems to be considerably greater in the rat than it is in the dog and in man A probable explanation of this species difference might lie in differences in intestinal secretion or reabsorption of the vitamin The occasional 100 % urinary recovery of injected riboflavin (STRIPP 1965) indicates that, in man, intestinal secretion may not be an important route of elimination

Interactions with proteins.

Table 2 indicates that most of the endogenous blood flavin is localized in the red blood cells However, when excess riboflavin is administered, the flavin concentration increases abruptly in the plasma, but only slightly in the blood cells (STRIPP 1965) The increase in the plasma concentration is solely due to free riboflavin If FMN is administered parenterally, both riboflavin and FMN are increased in the blood plasma (CHRISTENSEN 1969a, JUSKO *et al* 1970a)

The distribution of *endogenous* flavins among plasma proteins has been studied in humans by BAKER *et al* (1967) These workers reported that of the flavins precipitated by the Cohn procedure, 72 % was associated with

the globulins, 18 % with the β-lipoproteins and 10 % with the albumin. However, no information was given as to the amount of total plasma flavins bound to proteins, and the flavins were not individually analyzed

Interactions *in vitro* between flavins and plasma proteins have been studied in man, in the dog and in the rat JUSKO & LEVY (1969) showed that, in man, both riboflavin and FMN interact mainly with albumin, the total fractions bound at 30° being approximately 40 % and 80 %, respectively Flavin-albumin association constants were calculated to be 1 3 × 10³ and 3 2 × 10⁴ l/mole, respectively It is of interest that the flavin association constants to a tissue flavoprotein ("the old yellow enzyme") are at least 1,000 times greater (THEORELL & NYGÅRD 1954), since it indicates that the retention of flavins in the body is due to their high affinity to flavoprotein enzymes rather than to plasma proteins The binding of riboflavin to plasma proteins has been determined to 19 % in the dog (JUSKO *et al* 1970c) and 21 % in the rat (CHRISTENSEN 1971a) In all three species the extent of binding to plasma proteins is independent of the plasma riboflavin concentration over a wide range (in the rat, almost 100 fold) There is thus no evidence that the binding is saturated at high plasma riboflavin levels

The relatively weak binding of riboflavin to mammalian plasma proteins may be expected to have little influence upon the distribution and elimination of administered vitamin However, since the binding of flavins to plasma proteins has not been satisfactorily investigated at concentrations approaching physiological levels, interactions – of possibly low capacity – with plasma proteins other than albumin cannot be excluded The results of BAKER *et al* (1967) suggest that, on the whole, endogenous plasma flavins are attached to globulins rather than to albumin The fact that normally neither FAD nor FMN are present in the urine may suggest extensive binding of at least these flavins at physiological levels Such a binding would be of considerable importance for the physiological retention of the vitamin

There is at least one example of the binding of riboflavin to a specific carrier protein in the plasma being of vital importance, namely the single comb white leghorn chicken A genetic disease in a mutant strain of this species manifests itself in massive urinary loss of the vitamin at the expense of its deposition in the developing eggs Although the affected females do not become deficient, their progenies usually die in deficiency on approximately the 13th day of incubation A genetic defect in the renal tubular reabsorption of the vitamin was previously believed to cause the disease (cf the term riboflavinuria, COWAN *et al* 1966), but it has now been demonstrated that it is due to the lack of a riboflavin binding protein in the blood serum of the females and in their eggs (WINTER *et al* 1967) This specific protein, which has been characterized as a glycoprotein containing 8 S-S-bridges (PHILLIPS 1969, CLAGETT 1971), apparently possesses a unique

physiological function in the transfer of vitamin into the developing progenies

Transport across various membranes.

The transfer of vitamin across specific epithelial membranes is discussed elsewhere (intestinal transport in chapter 1, renal and biliary transport in chapter 4) Some of these transports include active or carrier mediated processes, enabling the vitamin to pass rapidly through complex membranes, which would otherwise have acted as barriers to its migration As a rule, however, the riboflavin molecule penetrates biological membranes slowly This is exemplified by the low permeability of intestinal preparations *in vitro* and by the absence of back diffusion in the kidney Moreover, the slow equilibration of free riboflavin between blood plasma and cells (STRIPP 1965) suggests a low permeability of cell membranes to the vitamin

Considering its poor ability to penetrate biomembranes and its rapid elimination, riboflavin, administered as a single dose, is unlikely to attain equilibrium among all body compartments until a main fraction has been eliminated The biexponential plasma elimination curves seen in functional anephric patients (JUSKO *et al* 1970b), and the triexponential ones observed in normal subjects (JUSKO & LEVY 1970), also appear to indicate that this *is not the case The data might be interpreted by a multicompartment open* model with a central compartment and a tissue compartment The second "slow" elimination component might be attributed to elimination from the slowly equilibrated tissue compartment, while the third elimination component may be explained by saturable renal reabsorption of the vitamin (JUSKO & LEVY 1970) The volume of the central compartment, estimated to be 16% of the body weight in the dog and in man, equals the extracellular water volume Although it is by no means certain that the readily equilibrated compartment represents this particular fraction of the body water, it indicates that the vitamin is rapidly distributed into some extravascular compartment Thus, the capillary membrane constitutes no barrier to its migration

The literature gives no dato on the transport of vitamin across the blood-brain barrier FOLEY *et al* (1967) studied the transport and storage of riboflavin-^{14}C in the retina of the rat, and reported a maximum radioactivity 45–60 minutes after intraperitoneal injection, i e an uptake rate comparable to that of the liver The passage of endogenous flavins across the human *placenta* was studied by LUST *et al* (1954) who found that the concentration of free riboflavin was four times greater in foetal than in maternal serum, whereas the concentration of FAD in foetal serum was only half of that in maternal serum Since there is apparently no difference between the binding characteristics of maternal and foetal plasma (GOLDSTEIN *et al* 1969), the

concentration gradient of riboflavin between the foetal and maternal blood suggests the presence of an active transfer mechanism in the placenta.

Exchange, incorporation and turnover.

If tracer doses of radioactive riboflavin are administered to animals, the radioactivity present in the tissues will reflect the exchange of radioactive molecules for unlabelled molecules, rather than the distribution of administered vitamin *per se* Thus, the rat liver, which has a rapid flavin turnover, retains approximately 25 % of the injected radioactivity after 24 hours, although all the excess riboflavin is then eliminated (YANG & MCCORMICK 1967, FOLEY *et al* 1967) The rapid incorporation of riboflavin into the liver has also been demonstrated by chromatographic scanning of radioactivity in liver extracts (CHRISTENSEN 1971b) After 90 minutes, 10 % of the injected radioactivity was recovered in the liver, mainly in the form of FMN and FAD, but no increase in the total flavin contents could be detected

Flavin turnover rates have been determined in whole animals YANG & MCCORMICK (1967) reported an average half life of 16 days in the rat, determined from the excretion of incorporated radioactivity in the urine This is approximately the same as that found by other investigators (AMOS *et al* 1966) There is evidence that the turnover rate is directly affected by the level of dietary riboflavin, since a much slower turnover rate has been reported for rats fed on a riboflavin deficient diet (FAULKNER & LAMBOOY 1961)

Chapter 3

Metabolism

Once absorbed, the riboflavin molecule is exposed to various metabolic changes These may be divided into *synthetic* pathways, leading to the active coenzyme forms of the vitamin, and *degradative* pathways, resulting in loss of vitamin activity Under steady state conditions, the biosynthesis of flavin coenzymes is counterbalanced by reverse hydrolytic reactions, which split up the coenzymes into free riboflavin As long as these processes proceed at equal rates, they do not influence the total storage or the elimination of the vitamin The degradative pathways, on the other hand, involve irreversible changes in the molecule, thus contributing to its elimination

Reversible synthesis of flavin coenzymes.

The synthetic and hydrolytic steps between riboflavin and its coenzyme forms are summarized in fig 5, which also indicates the enzymes involved and the binding of flavins to flavoproteins Conversion of riboflavin to the 5′-monophosphate ester, FMN, occurs in several tissues and is mediated by

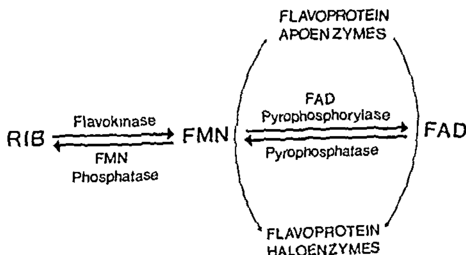


Fig 5 Synthetic and hydrolytic steps in the metabolism of riboflavin (RIB) and its coenzyme forms, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) Enzymes catalyzing each conversion and the interactions of FMN and FAD with flavoproteins are indicated

the ATP requiring enzyme flavokinase (KEARNEY & ENGLARD 1951) The flavokinase activity of rat tissues has been reported to decrease in the following order Liver, kidney, brain, heart, intestine (McCORMICK 1961) The conversion of FMN to flavin adenine nucleotide (FAD), which accounts for approximately 80 % of the tissue flavin, is mediated by FAD pyrophosphorylase, another enzyme which is present in the liver (RIVLIN 1970a) and in other tissues Hydrolysis of FAD to FMN by nucleotide pyrophosphatase (KORNBERG & PRICER 1950) and subsequent hydrolysis of FMN to riboflavin by phosphatases occur in most tissues At least two types of phosphatase contribute to the dephosphorylation of FMN An acid phosphatase, localized in the same tissues as flavokinase (McCORMICK 1961, McCORMICK & RUSSEL 1962), and an alkaline phosphatase, present in the intestine (OKUDA 1958b, CHRISTENSEN 1969b)

In the tissue, FMN and FAD combine with their respective flavoprotein apoenzymes to form stable haloenzymes Since unbound FAD has been shown to be much more susceptible to enzymatic hydrolysis than is bound FAD (KORNBERG & PRICER 1950), coenzymes synthesized in amounts exceeding the binding capacity may be rapidly split up again into riboflavin. For this reason, a relatively low concentration of unbound flavins in the tissues might be expected. This is consistent with the very low levels of ultrafiltrable flavins present in the blood plasma

Intravenously administered riboflavin does not cause any increase in the coenzyme forms in human blood plasma (STRIPP 1965), which suggests that equilibrium favours the presence of dephosphorylated vitamin in the non-tissue bound flavin pool Similarly, if FMN is injected intravenously, it becomes rapidly, although not completely, converted to free riboflavin in the circulating blood. The FMN riboflavin concentration ratio will decrease to approximately 1 3 (CHRISTENSEN 1969c, JUSKO *et al* 1970a), apparently reflecting an equilibrium between dephosphorylation and phosphorylation Although *in vivo* dephosphorylation is likely to occur mainly in the liver, it is noticeable that FMN may be dephosphorylated in blood *in vitro* by the action of an erythrocyte phosphatase (JUSKO *et al* 1970a, NOGAMI *et al* 1970)

Thyroid hormone appears to regulate hepatic levels of FMN- and FAD-requiring enzymes, which have been found decreased in hypothyroidism and increased in hyperthyroidism (RIVLIN & LANGDON 1966) Accumulating evidence suggests that thyroid hormone acts on two steps in the metabolism of flavoprotein enzymes By accelerating the synthesis of FMN and FAD, and by accelerating the flavoprotein synthesis The effect of thyroid hormone on the coenzyme synthesis seems exerted primarily on the flavokinase activity, the latter being increased by thyroid hormone and decreased in hypothyroidism. Biochemical similarities between riboflavin deficiency and hypothyroid-

Chapter 3

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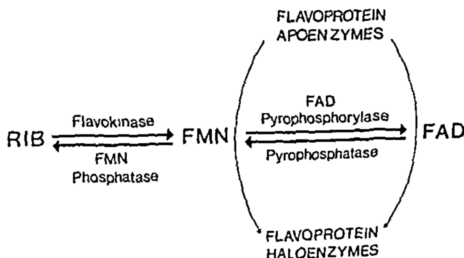


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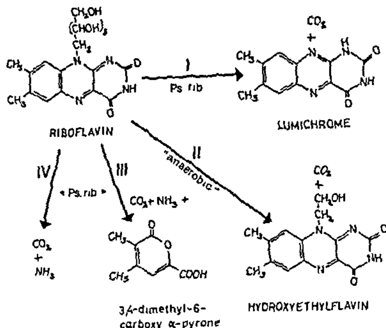


Fig 6 Pathways of microbiological degradation of riboflavin. Different strains of the soil bacteria *pseudomonas riboflavinus* are responsible for degradations according to scheme I III and IV Pathway II is due to an anaerobic soil bacteria Many intermediate compounds have been detected, but only terminal metabolites are indicated in the figure

to the monocyclic compound 3 4 dimethyl 6-carboxy α pyrone, carbon dioxide and ammonia (III), and another proceeding throughout into carbon dioxide and ammonia (IV) In addition to the terminal metabolites indicated in the figure many intermediate compounds have been detected, and some have been identified It has been suggested that the initial step in pathway III involves a mixed function oxygenase enzyme (HARKNESS *et al* 1964)

Degradation in the rat A survey of the literature shows several data suggesting decomposition of riboflavin in mammals However, until recently, *there has only been indirect evidence*, which *may explain* why several authors hold the view that riboflavin is *not* degradated in mammalian tissues (YAGI 1966 YANG & MCCORMICK 1967, OWEN & WEST 1970, RIVLIN 1970b) More recent studies give direct evidence of degradation of the vitamin in rats (CHRISTENSEN 1971b, c) thereby justifying renewed discussion

Indirect evidence of degradation mainly originates from incomplete re-

ism suggest that certain aspects of hypothyroidism may reflect functional forms of riboflavin deficiency (cf RIVLIN 1970b)

Continuous administration of glucocorticoids to humans has been reported to increase blood levels and urinary excretion of riboflavin given as parenteral loading doses Adrenalectomy as well as continuous administration of glucocorticoids caused a decrease in the flavin levels of the rat liver (NAKAMURA *et al* 1970) The significance of these findings is not clear at present However, all together they suggest that riboflavin metabolism is to some extent controlled by endocrine systems

Degradation

Although several flavins can replace riboflavin in the metabolism of certain strains of microorganisms, only riboflavin (6,7-dimethyl-9-(D-1′-ribityl)-isoalloxazine) satisfies the specific structural requirements of all mammalian flavoproteins (cf LAMBOOY 1966) Any irreversible change in the riboflavin molecule will therefore lead to partial or complete loss of its vitamin like activity When such chemical changes occur in the body, they are referred to as *degradation* processes, although – in theory – loss of activity may not necessarily involve chemical decomposition However, the fact that inactive synthetic metabolites have never been demonstrated, suggests that biological degradation of the vitamin implies decomposition in a chemical sense

While degradation of the riboflavin molecule is well known in certain microorganisms, controversy prevails with regard to its occurrence and significance in mammals Degradation in microorganisms have probably some characteristics in common with degradation in mammals, and may further directly contribute to the latter through the intestinal microflora For these reasons, the main features of microbiological degradation will shortly be reviewed

Degradation by microorganisms. The degradative pathways known in bacteria involve oxidation of the ribityl side chain as well as decomposition of the isoalloxazine structure (fig 6) A strain of *pseudomonas riboflavinus* an aerobic soil bacteria which utilizes riboflavin as its sole source of energy oxidizes the ribityl group to carbon dioxide (I), leaving the isoalloxazine portion intact in the form of lumichrome (cf FORSTER 1944) An enzyme catalyzing the initial step in this pathway, i e the hydrolytic cleavage of riboflavin into lumichrome and ribitol, has been isolated (YANAGITA & FORSTER 1956) Lumichrome is also known as a photolytic decomposition product of the vitamin (cf HEMMERICH *et al* 1965) Under anaerobic conditions another soil bacteria oxidizes riboflavin to 6,7-dimethyl 9 (2′-hydroxyethyl) isoalloxazine (II), also referred to as hydroxyethylflavin or "ethanol flavin" (MILES & STADTMAN 1955) A different strain of *ps riboflavinus*

MICK, however, used initial extraction procedures, which might have removed any possible degradation products FAULKNER & LAMBOOY *did not state the* paper chromatographic system used From our studies it appears that only certain systems separate riboflavin *from its degradation products.*

In the rat, the extent of degradation has been shown to depend on the *method of administration* (CHRISTENSEN 1971b) Much higher fractions of metabolites are present in the urine, if the vitamin is supplied orally or given subcutaneously as a suspension, than if administered intraperitoneally or subcutaneously in the form of dissolved vitamin This observation may reflect saturation of the metabolizing enzymes at the high peak levels produced by the rapid absorption routes However, another possible explanation is that degradation may be disfavoured following rapid absorption (producing short availability) because of slow migration of the vitamin into the metabolic sites Quantitatively, degradation accounted for up to 22 % of large oral doses of riboflavin in 24 hours (CHRISTENSEN 1971b) This figure is smaller than previously determined when using similar doses of unlabelled FMN (CHRISTENSEN 1969b) However, it is considered to be a more reliable estimate since it was obtained as the difference between the recovered radioactivity and the flavin fluorescence Radioactive metabolites excreted in the urine within 24 hours accounted for 50–80 % of the totally excreted radioactivity and 2–8 % of the administered radioactivity The fraction of metabolites in the faeces was usually lower, and considerable amounts of metabolites remained unexcreted after 24 hours However, the radioactivity of tissue extracts was too low to allow detection of tissue metabolites by the technique used

The nature of the major degradation products has not been determined, but some of their physico-chemical properties have been described (CHRISTENSEN 1971c) Besides small amounts of radioactive lumichrome carbon *dioxide and urea four unidentified radioactive metabolites* were found in urine These products were characterized by chromatographic R_f-values, lack of "flavin fluorescence", low lipid water partition coefficients and resistance to acid or enzymatic hydrolysis From these results it may be concluded that the metabolic processes involve decomposition of the tricyclic structure, and that none of the metabolites are conjugates of the riboflavin molecule Since only fragments containing the 2 positioned carbon atom in the molecule could be traced, the actual number of metabolites might well be greater than demonstrated at present

Site of degradation. Significance of the intestinal ***microflora.*** *The fact that* several strains of microorganisms can decompose riboflavin to fluorescent as well as to non fluorescent products gives rise to the question whether the degradation in rats, and probably in other species, is mediated by the intestinal microflora.

covery of administered vitamin. SURE & FORD (1943) reported that only 35 % of the vitamin given over a longer period could be recovered from the urine and faeces, and that tissue storage did not account for the remaining part. Since destruction of riboflavin could be detected on incubation with various tissue preparations *in vitro*, it was concluded that the fraction of the vitamin, not accounted for *in vivo*, was decomposed in the body. SELYE (1943), who administered heroic doses of riboflavin intravenously to nephrectomized rats, suggested that its disappearance from the tissues was due to destruction in the body. Twenty-four hours after the administration of 500 μg of riboflavin intraperitoneally, BESSEY *et al.* (1958) recovered only 25 % in the urine, faeces and carcass. They reported that the rate of degradation may vary from 0.04 μg/g body weight/24 hours in deficiency to 6 μg/g body weight/24 hours during excessive administration. YANG & MCCORMICK (1967) were unable to reproduce their findings and considered the low recoveries erroneous. However, our studies (CHRISTENSEN 1969b) have confirmed the results obtained by previous investigators, as more than 50 % of large doses of FMN could not be accounted for in the urine, faeces and carcass.

More direct evidence of degradation comes from studies with radioactive isotopes of the vitamin. FAULKNER & LAMBOOY (1961) reported that only part of the radioactivity excreted in the urine of animals with riboflavin-2-^{14}C incorporated in their tissues was due to microbiologically active vitamin. However, radio-autograms of urine revealed only traces of an unidentified metabolite. One hour after subcutaneous injection of a large radioactive dose, YAGI *et al.* (1966) found only riboflavin and traces of FMN in the urine. YANG & MCCORMICK (1967) administered small amounts of riboflavin-2-^{14}C intraperitoneally and determined the paper chromatographic distribution of radioactivity in the urine, faeces and tissue extracts after 24 hours. Riboflavin was reported to account for 70–95 % of the total radioactivity, but no metabolites were detected except for trace amounts of lumichrome and lumiflavin in the faeces. However, in recent studies by the author (CHRISTENSEN 1971b, c) significant amounts of radioactive degradation products were detected in the urine and faeces of rats following administration of riboflavin-2-^{14}C.

It might be of interest to state some possible reasons for the discrepancy between our results and those of previous investigators. YAGI *et al.* (1966) and YANG & MCCORMICK (1967) administered the vitamin parenterally only, which according to our experience, results in rapid excretion of unchanged vitamin without any appreciable amounts of metabolites in the urine (CHRISTENSEN 1971b). The experiments of YAGI *et al.* (1966) were probably too short term to demonstrate any decomposition. In our study, the radioactive samples were applied directly to the chromatographic paper. YANG & MCCOR-

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Site of degradation Significance of the intestinal microflora. The fact that several strains of microorganisms can decompose riboflavin to fluorescent as well as to non fluorescent products gives rise to the question whether the degradation in rats and probably in other species, is mediated by the intestinal microflora.

Degradation of riboflavin by intestinal microorganisms *in vivo* and *in vitro* has been reported in the rat (HOTTA & ISHIGURA 1955, INNAMI *et al* 1965), in ruminants (OLCESE & PEARSON, 1948, HOBSON *et al* 1969) and in man (SAKKAI & HAMADA 1960) On the other hand, neither YANG & MCCORMICK (1967) nor CHRISTENSEN (1969b) found significant degradation of riboflavin incubated with rat faeces *in vitro* These findings are difficult to interpret for two reasons Firstly, it is possible that tissue metabolites *in vivo* may be secreted into the intestinal tract, and secondly the intestinal microflora may change characteristics when cultivated *in vitro* (SCHELINE 1968) Furthermore, in the rat, differences in diets may result in variations Thus, HOTTA & ISHIGURA (1955) reported a greater decomposition following animal diet than following vegetable diet

It is noteworthy, however, that only fluorescent metabolites such as lumichrome and lumiflavin have been reported to result from decomposition by *intestinal* microorganisms, and that only few reports present quantitative data Thus, although in the rat some riboflavin is undoubtedly decomposed to isoalloxazine compounds by intestinal bacteria, there is no evidence that the *formation* of non-isoalloxazine metabolites is mediated by the intestinal microflora The fact that degradation occurred to the same extent whether the vitamin was administered orally or parenterally (i e, subcutaneously as a suspension), and that the fraction of metabolites was generally much higher in the urine than in faeces (CHRISTENSEN 1971b) gives further support to the assumption that these metabolites are produced in the tissues On the other hand lumichrome, which is presumably produced by intestinal microorganisms, occurred to a much higher extent in the faeces Finally, SURE & FORD (1943) reported that *in vitro* appreciable amounts of riboflavin may be decomposed if incubated with liver, spleen, lung, heart and intestine of the rat YANG & MCCORMICK (1967) reported that no metabolites were produced in homogenates of various rat tissues, but only chloroform-soluble compounds were apparently assayed

The main site of degradation in the rat body has not been clearly established CHRISTENSEN (1969d) showed that the elimination from the plasma was considerably decreased in animals without blood circulation through the liver However, a recent report (NOGAMI *et al* 1970) suggests that biliary secretion is more extensive than previously believed, so it is difficult to evaluate the extent to which impaired biliary secretion might have contributed to this finding Still, the very slight decrease in plasma levels in animals without hepatic circulation suggests that, *in vivo*, extrahepatic tissues at the most contribute little to degradation SURE & FORD (1943), however reported a greater *in vitro* metabolizing activity in the lung and small intestine than in the liver Further studies on isolated tissues or tissue fractions are required to elucidate this problem

Degradation in other species. *Our knowledge as regards the metabolic fate of riboflavin in other mammals is very limited* Radioisotopes have, as far as is known, not been used in metabolic studies in larger animals or man Similarly, recovery experiments requiring *total body analyses* have been limited to small animals

Small amounts of fluorescent metabolites have been detected in the urine *and milk in ruminants following administration of very large oral doses of* the vitamin (OWEN & WEST 1970) Two of the metabolites have been identified as 6,7-dimethyl 9 (2-hydroxyethyl) isoalloxazine ("hydroxyethylflavin") *and 6,7-dimethyl 9-(formylmethyl) isoalloxazine* ("formylmethylflavin"), i e isoalloxazine compounds produced by oxidations in the ribityl side chain Such products are similar to those known to occur from bacterial degradation (see fig 6), and their formation in ruminants (i e sheep, goats and calves) is presumably due to the activity of the ruminal microflora (OWEN & WEST 1970) Thus, they are not produced following the subcutaneous injection of riboflavin or if riboflavin is incubated with various tissues *in vitro*

Similar products were detected in the urine of the rabbit and of man, but were absent in the urine of the dog. In human urine hydroxyethylflavin was the predominant metabolite, accounting for up to 0 6 % of the ingested, and 16 % of the excreted isoalloxazine (WEST & OWEN 1969) The fact that such metabolites occurred in the urine only after massive doses of the vitamin and that they were not excreted within the first 24 hours after administration, suggests that they are produced by the intestinal microflora The analytical procedures used by Owen and co-workers were unsuitable for the detection of non fluorescent metabolites similar to those found in the rat

The significance of degradation with regard to the elimination of riboflavin in other mammals than the rat has to be further clarified In man, the high urinary output following oral administration and the occasional complete *recovery following parenteral administration* (STRIPP 1965, JUSKO & LEVY 1967) suggest that degradation may only contribute little to the elimination of excess vitamin Furthermore, the fact that humans on riboflavin restricted diets over longer *periods can excrete more vitamin* than is ingested (NAJJAR *et al* 1944), suggests that – under these conditions – degradation is superimposed by intestinal biosynthesis

Chapter 4

Excretion

Riboflavin is excreted unchanged in the urine, faeces and – to a negligible extent – by other routes. The predominant excretion route depends on the dose, method of administration and species. At very low dietary vitamin intakes, excretion in the urine and faeces decreases to a minimum level. It will, however, never reach zero, even if riboflavin is absent in the diet and symptoms of deficiency have developed. On the other hand, in an adequately supplied organism excess riboflavin is rapidly excreted as it cannot be stored in the tissues. This chapter deals mainly with the kinetics and mechanisms of the excretion processes. In addition, the general interpretation of excretion data will be briefly discussed.

Excretion with urine.

The fraction of orally administered riboflavin excreted in the urine, varies considerably from species to species. For doses well exceeding the daily requirement, 40–60 % is usually recovered from the urine in man (see below), in the monkey (COOPERMAN *et al.* 1945), the dog (POTTER *et al.* 1942) and the horse (PEARSON *et al.* 1944), whereas the corresponding figures in the rat are 5–10 % (SURE & FORD 1943, CHRISTENSEN 1969a), and in the goat only 1 % (OLCESE & PEARSON 1948). However, as will be discussed, these differences appear to reflect species variations with respect to absorption, distribution or extrarenal elimination, rather than to renal excretion *per se*.

The influence of the absorption rate on the amount of vitamin excreted in the urine may be exemplified by experiments in the rat (CHRISTENSEN 1969c). Here, the urinary recovery of comparable doses was found to be 82 % following intravenous injection, 50 % following slow intravenous infusion, 29 % following intraperitoneal injection and 7 % following oral ingestion. It appears from these figures that the greater the assumed absorption rate, the more unchanged vitamin is excreted in the urine. An opposite relationship exists between rate of absorption and degradation (cf. chapter 3). Considered together, the data suggest a shift in elimination from urinary excretion towards degradation if the absorption, i.e. the availability in the

body, is prolonged Possible explanations for this relationship have been discussed in chapter 3

Interpretation of urinary excretion data. The relationship between intake and urinary excretion of riboflavin has been extensively studied, and there has been much discussion concerning the interpretation of the data on urinary excretion Experiments in man indicate that, independent of size, oral doses ranging from 1–20 mg result in urinary excretions of 40–60 % (MELNICK *et al* 1945, MORRISON & CAMPBELL 1960, STRIPP 1965, LEVY & JUSKO 1966a) Oral doses exceeding 20 mg are, however, excreted to a lesser extent (STRIPP 1965, LEVY & JUSKO 1966a) This phenomenon has been attributed to limited intestinal absorption as discussed in chapter 1 The excretion of *dietary* vitamin appears to be of similar magnitude if the intake exceeds the requirement (BREWER *et al* 1946) If, however, the intake falls to below normal requirement (i e approximately 2 mg per day in man), a smaller fraction is excreted in the urine (BREWER *et al* 1946, BRO-RASMUSSEN 1958) On an empirical basis the critical intake above which a greater percentage of the vitamin appears in the urine has been used as an indicator for the optimum requirement of the vitamin This practice seems justifiable because of its agreement with the optimum requirements as estimated by different methods (cf BRO-RASMUSSEN 1958) The coincidence of critical intake and optimum requirement, estimated by using tissue saturation as a criterion, indicates that a greater fraction of administered riboflavin is excreted in the urine when the tissues become saturated

A possible explanation of the shift in elimination towards urinary excretion on approaching tissue saturation appears from fig 7, which shows the plasma and urinary levels of free riboflavin in rats supplied with various amounts of the vitamin At low intakes, the excretion is insignificant, suggesting tubular reabsorption if the associated plasma levels are taken into account. At a certain point (the critical point) the urinary excretion increases more abruptly than the plasma concentration suggesting saturation of tubular reabsorption In other words, the higher urinary recovery of vitamin given in excess of the critical intake may be attributed to saturation of its renal reabsorption at the plasma levels associated with tissue saturation Interestingly a similar interpretation of the critical intake was advanced (BRO-RASMUSSEN 1958) several years before it was recognized that riboflavin is actually reabsorbed in the kidney

The relationship between the nutritional state as regards riboflavin and its excretion in the urine has attracted much attention because of the absence of an easily detectable deficiency syndrome However, attempts to use urinary excretion as a diagnostic tool have failed in so far as no reliable excretion test has been developed (AXELROD *et al* 1941, LOSSY *et al* 1951, CLARKE 1969) For this purpose the spontaneous excretion is of little value since it

Chapter 4

Excretion

Riboflavin is excreted unchanged in the urine, faeces and – to a negligible extent – by other routes. The predominant excretion route depends on the dose, method of administration and species. At very low dietary vitamin intakes, excretion in the urine and faeces decreases to a minimum level. It will, however, never reach zero, even if riboflavin is absent in the diet and symptoms of deficiency have developed. On the other hand, in an adequately supplied organism excess riboflavin is rapidly excreted as it cannot be stored in the tissues. This chapter deals mainly with the kinetics and mechanisms of the excretion processes. In addition, the general interpretation of excretion data will be briefly discussed.

Excretion with urine.

The fraction of orally administered riboflavin excreted in the urine, varies considerably from species to species. For doses well exceeding the daily requirement, 40–60 % is usually recovered from the urine in man (see below), in the monkey (COOPERMAN *et al.* 1945), the dog (POTTER *et al.* 1942) and the horse (PEARSON *et al.* 1944), whereas the corresponding figures in the rat are 5–10 % (SURE & FORD 1943, CHRISTENSEN 1969a), and in the goat only 1 % (OLCESE & PEARSON 1948). However, as will be discussed, these differences appear to reflect species variations with respect to absorption, distribution or extrarenal elimination, rather than to renal excretion *per se*.

The influence of the absorption rate on the amount of vitamin excreted in the urine may be exemplified by experiments in the rat (CHRISTENSEN 1969c). Here, the urinary recovery of comparable doses was found to be 82 % following intravenous injection, 50 % following slow intravenous infusion, 29 % following intraperitoneal injection and 7 % following oral ingestion. It appears from these figures that the greater the assumed absorption rate, the more unchanged vitamin is excreted in the urine. An opposite relationship exists between rate of absorption and degradation (cf. chapter 3). Considered together, the data suggest a shift in elimination from urinary excretion towards degradation if the absorption, i.e. the availability in the

for those of BURCH *et al* 1948), on the elucidation of the excretion mechanism at physiological plasma levels Studies carried out at approximately 100 fold higher plasma levels indicate that, following intravenous infusion, the urinary excretion rate decreases exponentially as a function of time, at least during the first few hours Half lives for excretion rates were found to be 1 2 hours in man (LEVY & JUSKO 1966b), and 0 4 hours in the rat (CHRISTENSEN 1969c) In man, the exponential decrease in the excretion rate is also observed following oral administration (LEVY & JUSKO 1966a), whereas the kinetics following rapid intravenous injection is more complicated (JUSKO & LEVY 1970)

Tubular secretion. The exponential decrease of the excretion rate may suggest a first order excretion kinetics over a wide range of plasma concentration compatible with glomerular filtration However, in man (LEVY & JUSKO 1966b) as well as in the rat (CHRISTENSEN 1969c), approximate estimates of the renal clearance exceeded the glomerular filtration rate, and recent clearance experiments have accordingly established that tubular secretion occurs in man (JUSKO *et al* 1970a), in the dog (JUSKO *et al* 1970c) and in the rat (CHRISTENSEN 1971a) Tubular secretion of riboflavin has previously been demonstrated in the chicken (RENNICK 1960)

Comparative excretion data indicating tubular secretion in the four species are shown in table 4 The riboflavin inulin clearance ratios indicate that the riboflavin clearance may exceed the glomerular filtration rate (GFR) by

Table 4

Quantitative data on renal excretion of riboflavin in 4 different species. All data have been obtained at plasma riboflavin levels above 0 3 µg/ml The clearance ratios have not been corrected for the binding of riboflavin to plasma proteins

Species (number of individuals)	Riboflavin / Inulin clearance ratio: individual range	Riboflavin / Inulin clearance ratio: minimum value after probenecid	Riboflavin / PAH[1] clearance ratio	Authority
Man (2)	2.6–3 1 [2]	1 2		JUSKO *et al* 1970a
Dog (5)	0 9–2 0	0 7	0 41–0.55	JUSKO *et al* 1970c
Rat (22)	1 2–3 2	0 8		CHRISTENSEN 1971a
Chicken (12)			0 27–0 54 [3]	RENNICK 1960

[1] Paraaminohippuric acid
[2] Riboflavin to creatinine clearance ratio
[3] Ratio between the apparent tubular excretion fraction (ATEF) of riboflavin and paraaminohippuric acid

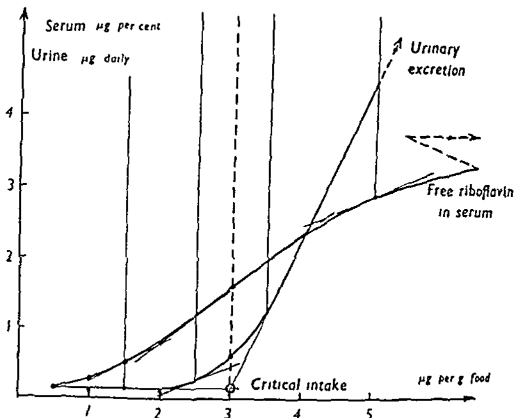


Fig 7 Urinary *excretion of riboflavin, and serum concentration of free riboflavin,* as influenced by the dietary vitamin intake in the rat The *critical point of urinary ex cretion,* i e. the turning point at which a given increase of intake gives a relatively *higher increase* of urinary excretion than of free riboflavin in the serum has been *determined* by graphical differentiation The figure has been reproduced from BRO-RASMUSSEN 1958 with the permission of the author The data are from BURCH *et al* 1948

reflects the immediate vitamin intake rather than the body levels By means of appropriate test doses it has been possible to detect unusually high retentions of the vitamin in deficiency states However, large variations in normal subjects render it impossible to define an "abnormal" response (DAUBENMERKL 1947) Evaluation of the nutritional state by means of urinary excretion tests is further complicated by the fact that retention of the vitamin, and hence its excretion in the urine, is highly dependent on changes in the protein balance (BRO-RASMUSSEN 1958) This relationship, i e higher retention during a positive nitrogen balance and *vice versa,* reflects that retention of riboflavin is dependent on the amount of available flavin-binding proteins

Kinetics of renal excretion. For the purpose of studying the renal excretion processes *per se* (i e uninfluenced by absorption, distribution or extrarenal elimination processes), the concentration of free riboflavin in the plasma should be determined This difficulty may explain the absence of data (except

Thus, the fraction of the excreted riboflavin secreted by the tubulus remains unchanged at plasma riboflavin levels up to 2, 5 and 20 µg/ml in man, in the dog and in the rat, respectively In the rat, riboflavin clearances could not be determined at higher plasma levels because of precipitation of riboflavin in the kidneys (CHRISTENSEN 1971a)

Mechanism of tubular secretion. The nature of the active transport of riboflavin from blood to urine is unknown Most compounds secreted by the renal tubules are present, partly or completely, in an ionized form at physiological pH, and the electrical charge is thought to be essential for the transport (cf Goldstein *et al* 1969) Therefore, the fact that riboflavin is present in the plasma exclusively as an unloaded molecule ($pS_I = 1\,9$, $pS_{II} = 10\,2$) is interesting with reference to the transport mechanism. The following possibilities might be considered Riboflavin may be secreted by a non ionic transport mechanism, which is possibly unique for the vitamin, or it may undergo biotransformation into an ionized metabolite that is able to enter one of the ionic transport systems As an attractive hypothesis related to the latter possibility, a renal phosphorylation dephosphorylation mechanism should be considered, as also suggested by JUSKO *et al* (1970c) This hypo-

[illegible]

and (iii) FMN is primarily, if not exclusively, excreted in the urine in the form of riboflavin (CHRISTENSEN 1969c, JUSKO *et al* 1970a) According to this hypothesis the blocking effect of probenecid might be attributed either to competition with anionic transport of FMN, or to inhibition of renal phosphorylation

Judging from stop-flow studies in the dog (JUSKO *et al* 1970c), the site of the tubular transport is approximately the same as that for PAH in the *proximal tubule* This, in addition to the fact that probenecid inhibits the clearance of riboflavin and PAH in a strictly parallel dose-dependent manner (JUSKO *et al* 1970c), suggests anionic transport However, the "papilla-to-cortex concentration profile" of FMN or total flavin in the dog kidney differs appreciably from that of PAH (JUSKO *et al* 1970c)

Tubular reabsorption. The data of BURCH *et al* (1948) suggesting reabsorption of riboflavin in the rat at physiological plasma levels were shown *in fig. 7* An earlier interpretation of these data (BRO-RASMUSSEN 1958) dealt with the apparent "threshold" for the vitamin in plasma as indicated *by an abrupt* increase in urinary excretion at a certain level However, the data also allow calculation of renal clearances *The* clearance-values associated with low intakes 8–20 ml/100 g body weight/24 hrs, indicate that, when compared with normal values of the GFR (1400 ml/100 g body weight/ 24 hrs) (BERGLUND *et al* 1969), tubular reabsorption must be almost com-

200 % in man and in the rat, and by 100 % in the dog Furthermore, these values should be compared with *filtration* clearances of less than the GFR, because of the binding of riboflavin to plasma proteins It is characteristic that the clearance ratio obtained in each species varies considerably from individual to individual In the rat, these variations were not related to variations of any known parameter and hence probably reflecting individual variations in secretion activity *per se* (CHRISTENSEN 1971a) According to JUSKO *et al* (1970c) variations in the dog might have been due to partial block of the secretion by paraaminohippuric acid (PAH) infused simultaneously

The maximum riboflavin inulin clearance ratios indicate that riboflavin is not cleared completely from the blood in any of the species Thus, the clearance of PAH, a substance that is almost completely cleared at low plasma levels, has been reported to be 5 0, 3 5 and 3 7 times greater than that of inulin in man, in the dog and in the rat, respectively (cf SMITH 1951) Also, the riboflavin PAH clearance ratios determined in the dog indicate that the riboflavin clearance is approximately half as great as that of PAH It has been suggested that for compounds with a low affinity to the tubular transport mechanism, protein binding may influence the extent of tubular secretion (cf WEINER 1967) However, on the basis of the present data it cannot be established whether the secretion is limited by low affinity or by protein binding

Chickens are very suitable for studying tubular secretion since in birds the tubules are separately supplied with blood from the renal portal system (SPERBER 1948a) The data referred to in table 4 were obtained by the technique of SPERBER (1948b), allowing a direct estimate of the amount secreted by the tubules As in mammals, only half of the riboflavin delivered to the tubules is secreted into the urine

Probenecid has been shown to inhibit tubular secretion of riboflavin in all the four species mentioned Table 4 indicates the minimum riboflavin inulin clearance ratios observed in man, in the dog and in the rat after probenecid administration Considering the binding to plasma proteins, the values obtained in the dog and in the rat (0 7–0 8) are in good agreement with those expected in lack of tubular secretion Since there is reason to believe that these values represent a complete block of secretion, their magnitude probably indicates that riboflavin is not reabsorbed to a measurable extent by passive diffusion The higher riboflavin inulin clearance ratio after probenecid in the humans reflects incomplete block of the secretion in the reported cases, since data from the same study indicate that probenecid may decrease the riboflavin clearance to values less than the GFR Tolazoline a compound known to participate in cationic tubular transport, has been shown to inhibit tubular secretion in the chicken (RENNICK 1960)

A Tm for the tubular secretion of riboflavin has not been demonstrated

riboflavin decreased spontaneously The riboflavin clearance *versus* plasma concentration curve from their study (fig. 8) may be interpreted as the result of bidirectional tubular transport Tubular secretion below the Tm, and tubular reabsorption with a low saturation threshold. The reabsorption rate *versus* plasma concentration curve derived from the data shown agreed reasonably well with the Michaelis Menten equation, and an apparent Tm for the reabsorption was calculated to 33 μg/min. A similar relationship between renal clearance and plasma concentration was found in the dog (JUSKO & LEVY 1970)

In the rat, it was not possible to demonstrate a decreased riboflavin inulin clearance ratio at low plasma levels (CHRISTENSEN 1971a), probably due to methodological limitations However, both in the rat (CHRISTENSEN 1971a) and in the dog (JUSKO & LEVY 1970), a positive correlation exists between urine flow and riboflavin clearance at the very low plasma levels The dependence of renal clearance on the tubular load *per se* suggests tubular reabsorption

Moreover, the earlier literature also contains indications of a correlation between urine volume and urinary excretion of riboflavin Thus, increased excretion of the vitamin has been reported in diabetic rats and in rats with osmotic diuresis (NIEMAN & JANSEN 1955) as well as in humans during water diuresis (JOHNSON 1946) LOSSY *et al* (1951) reported that diabetic patients excreted significantly more of a small test dose in the urine than did normal subjects, and that there was a positive correlation between urine volume and riboflavin excretion

Summary of renal excretion. To summarize, the renal handling of riboflavin involves glomerular filtration of unbound vitamin in the plasma (60–80 %) non saturable tubular secretion and saturable tubular reabsorption. The net clearance depends on the ratio between the bidirectional tubular transports and may vary with the plasma concentration At high levels (i e above 0 5 μg/ml) secretion pre dominates, and hence the clearance exceeds the GFR. Tubular reabsorption influences the net clearance only at very low (physiological) plasma levels Under such circumstances reabsorption may exceed secretion, so that the clearance becomes less than the GFR. As tubular secretion and reabsorption proceed simultaneously, it is unlikely that the vitamin could be reabsorbed completely under any condition This view is supported by the finding that riboflavin is present in the urine even if the intake is negligible and deficiency symptoms may have developed (FRAZER *et al* 1940 SEBRELL *et al* 1941, BURCH *et al* 1948, CHRISTENSEN 1969a) For this reason riboflavin cannot be considered a true threshold substance like for instance glucose

plete In other species, the lack of simultaneous determinations of riboflavin levels in plasma and in urine prevents calculation of exact "endogenous" clearance values However, from the plasma and urine data in adult volunteers on unrestricted diets (STRIPP 1965), approximate clearances of less than 67 litres/24 hrs can be calculated This figure is below the expected "filtration clearance" taking into account the known binding to the plasma proteins, and considerably less than the expected clearance in the presence of tubular secretion

One objection to the interpretation of these data as evidence of tubular reabsorption should be mentioned The extent of plasma protein binding at physiological plasma levels is not known Although studies *in vitro* indicate that only a constant, minor fraction is bound to albumin at high plasma levels, more extensive binding, of possibly limited capacity, may exist at low plasma concentrations (cf chapter 2) However, considering that protein bound vitamin is presumably available for tubular secretion, it seems unlikely that protein binding *per se* may explain the extremely low clearance values found at physiological plasma levels in the rat

Recent studies in man, in the dog and in the rat procure further evidence of tubular reabsorption of riboflavin JUSKO & LEVY (1970) reported one subject in whom the riboflavin clearance decreased to approximately the same level as the creatinine clearance when the concentration of injected

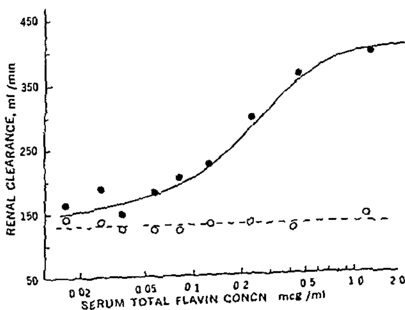


Fig 8 Renal clearance of total riboflavin (●) in a human subject as a function of decreasing serum flavin concentration after intravenous injection of 31 mg of FMN Open symbols (○) represent simultaneously obtained creatinine clearances Reproduced from JUSKO & LEVY 1970 with the permission of the authors

recently by NOGAMI *et al* (1970), who collected bile from the cannulated bile duct of the rat at various intervals following intravenous flavin injections Free riboflavin was the main flavin excreted in the bile no matter whether riboflavin, FMN or FAD had been administered Following very large doses (4 mg), 30 % was recovered from the bile, and 80 % of the totally excreted riboflavin appeared in the bile within 2 hours However, following small doses (40 µg) only 15 % was excreted in the bile, and the excretion was much more prolonged From these data it was suggested that different mechanisms operate in biliary secretion or riboflavin at high and at low plasma levels However, a possible influence of the dose on the distribution volume may have contributed to the differences observed

YAGI *et al* (1968), who injected 400 µg of radioactive vitamin subcutaneously into rats, recovered 10 % of the radioactivity from the bile collected over a period of 3 hours. The possible existence of radioactive metabolites was, however, not considered.

In man, biliary excretion of riboflavin has not been investigated by direct cannulation methods The main evidence of its existence is indirect, as discussed under enterohepatic circulation

Biliary secretion of riboflavin has character of an active transport from blood to bile (cf NOGAMI *et al* 1970), as is the case with other compounds undergoing biliary secretion (GOLDSTEIN *et al* 1969) The migration of riboflavin from the blood to hepatic cells and bile capillaries has been studied in the living rat by fluorescence microscopy (HIRT & WIMMER 1940) Few minutes after an intravenous injection of riboflavin, diffuse fluorescence is observed in the hepatocytes, and after 10 minutes fluorescence occurs in the bile capillaries The secreted vitamin seems to be present in the form of unchanged riboflavin (TEDESCHI 1955, NOGAMI *et al* 1970)

Mucosal secretion SELYE (1943) reported that, following intravenous injection of large doses to nephrectomized rats, riboflavin crystals occurred in the lumen of ligated duodenal and jejunal segments From the distribution of secreted vitamin he concluded that biliary secretion is quantitatively unimportant More recent studies similarly indicate intestinal secretion of the vitamin administered parenterally to rats (YAGI 1954a, YANG & MCCORMICK 1967 CHRISTENSEN 1969b) and rabbits (YAGI 1954b) Although the early experiments of SELYE (1943) were rather crude and carried out under unphysiological conditions with regard to doses and animals, our recent investigation of the problem (see page 31) resulted in essentially the same conclusions viz that secretion occurs from the entire small intestine, and that biliary secretion contributes little to this

Very little is known about the mechanisms of mucosal secretion of compounds In case of riboflavin the accumulation of the vitamin in the mucosal cells may be associated with the secretion. Thus, YAGI (1954b) observed that

Excretion in faeces.

Riboflavin present in the faeces originates from unabsorbed vitamin, from vitamin secreted into the intestinal tract or from vitamin synthesized by the intestinal microorganisms Only that part resulting from excretory processes, i e biliary or mucosal secretion, is considered to be excreted in a strict sense However, difficulties in distinguishing between the fractions originating from each of these sources necessitate the term "faecal excretion" to indicate the total loss of vitamin in the faeces In the following, faecal excretion in general and the influence of various factors will be treated first Secondly, the excretion processes *per se* will be considered

An obligatory loss of riboflavin occurs via the faeces, as in the urine Rats maintained on a riboflavin free diet excrete a low, constant amount by this route (FAULKNER & LAMBOOY 1961, CHRISTENSEN 1969a), indicating that the secreted or synthesized vitamin is not completely absorbed from the intestine The reason for this may be that secretion/biosynthesis occurs distally in the intestinal tract, or that flavin produced by bacteria is not readily available for absorption Young men on a vitamin intake restricted to 60–90 μg daily for 10 weeks excreted 5–6 times this amount in the faeces (NAJJAR *et al* 1944) As no sign of deficiency appeared during this period, the faecal output was thought to reflect intestinal biosynthesis rather than loss of vitamin from the body

In the rat, 10–25 % of orally administered riboflavin is recovered from the faeces (CHRISTENSEN 1969a, SURE & FORD 1943) The question whether the vitamin excreted in the faeces originates from unabsorbed or secreted vitamin may be of little importance, as an equilibrium between absorption and secretion is probably established during its passage along the gut Faecal excretion following oral doses of vitamin has not been systematically investigated in humans NAJJAR *et al* (1944) reported that intravenous injections of 5–20 mg of riboflavin did not cause any increase of the faecal excretion This may indicate that intestinal secretion, if any, is overcompensated by absorption As opposed to this, parenterally administered vitamin can be recovered from faeces in the rat (CHRISTENSEN 1971b)

Biliary secretion. Riboflavin has been reported to be present in the bile of man (DE PREUX 1942), of the dog (TEDESCHI 1955), the rabbit (DE PREUX 1942, MIZUHARA 1950) and the rat (HIRT & WIMMER 1940, YAGI *et al* 1968, NOGAMI *et al* 1970) However, only few reports include quantitative data allowing an estimate of the importance of biliary excretion in the overall elemination Furthermore, the data for man (DE PREUX 1942) were obtained by a duodenal cannulation technique which may also include mucosal secretion

The only kinetic study on biliary secretion of riboflavin was reported

Chapter 5

Conclusions and Pharmacokinetic model

In the foregoing chapters a number of pharmacokinetic processes, in which riboflavin participates, have been described and discussed in detail Our knowledge concerning these processes comes partly from studies in intact animals, partly from studies with animal preparations or from *in vitro* systems. In the living body, however, all these processes occur simultaneously and may be difficult to distinguish from each other It is thus difficult to determine for example the net intestinal absorption rate in intact animals since secretion also occurs simultaneously into the intestinal tract

This chapter is an attempt to summarize the conclusions reached in chapters 1–4, and to consider them in relation to the integrated mammalian organism For this purpose a simplified pharmacokinetic model has been suggested as shown in fig. 9 It should be emphasized, however, that this model takes into consideration the major pharmacokinetic processes only, and it does not pretend to offer an exhaustive description of the fate of the vitamin in the body

The kinetics and mechanisms of the *gastro intestinal absorption* seem to be different in certain animals and in man In the rat, for example, the vita min is absorbed slowly and incompletely from the gut, as compatible with passive diffusion considering the poor ability of the molecule to penetrate biomembranes. In contrast to this, there is a more rapid and complete absorption of the vitamin in man The absorption process exhibits characteristics of site specificity and saturability suggesting mechanisms other than passive diffusion The differences in kinetics are reflected by the plasma concentration *versus* time curves While in man, oral administration of riboflavin produces a definite temporary peak in the plasma concentration, the increase is much smaller and more prolonged in the rat. Absorption of riboflavin from *parenteral* sites has been studied very little However, in animals as well as in man parenteral administration results in higher urinary recovery than does oral administration

After being absorbed, riboflavin is *distributed* in the body fluids, from which it is removed by several processes Some of these processes are reversible establishing a dynamic equilibrium between free riboflavin in the body fluids and riboflavin bound to or located at various sites binding to plasma and tissue proteins, synthesis of the coenzyme forms FMN and FAD, which

in the rabbit, riboflavin is accumulated in the mucosa, primarily in the ileum, from which it migrates into the intestinal lumen

Enterohepatic circulation. Riboflavin secreted into the small intestine, directly or via the bile, would be expected to be available for reabsorption It may be difficult, however, to determine the actual extent of reabsorption An enterohepatic circulation of the vitamin has been suggested in man (JUSKO & LEVY 1967a) on the basis of the urinary excretion data following oral administration In certain cases the usual exponential decline in urinary excretion rate was superimposed by secondary maxima associated with meal times Since excretion maxima could be observed at meal times whether the vitamin was administered orally, parenterally or not at all (JUSKO & LEVY 1967b), a promoting effect of bile on the absorption of the vitamin could be ignored

NOGAMI *et al* (1970) reported that there was no difference between plasma elimination curves following riboflavin injections to rats with or without bile fistula However, this does not necessarily exclude enterohepatic circulation since mucosal secretion is possibly predominant and may mask the reabsorption of riboflavin secreted with the bile

Secretion into the intestine followed by reabsorption may prolong the availability of single doses of riboflavin YAGI (1954b) reported that in rabbits the plasma concentration of riboflavin remained high for a long time after single injections In the rat, however, the very short half-life of injected riboflavin suggests that reabsorption of secreted vitamin is of minor importance

Excretion by other routes.

Excretion by other routes than in the urine and faeces normally contributes little to the elimination of riboflavin Human sweat contains 0 12 (0 03–0 30) mg of riboflavin per litre (CORNBLEET *et al* 1943), which indicates that approximately 5 % of a standard intake of 2 2 mg may be excreted with the sweat

The concentration of riboflavin in milk is somewhat higher, i e 0 3 (0 2–0 8) mg per litre in man (MACY 1949) and 1 6 (1 2–2 0) mg per litre in the cow (DAHLBERG *et al* 1952) These levels are high as compared with the plasma riboflavin levels, probably reflecting binding of the vitamin to certain milk proteins (LEVITON & PALANSCH 1960) It may be calculated that lactating women excrete up to 0 8 mg of riboflavin per day via the milk, an amount necessitating an extra supply of the vitamin during lactation

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The binding of administered riboflavin to *plasma proteins* is not very extensive, and has possibly minor pharmacokinetic implications. The fraction bound in the plasma, mainly to the albumin, approximates 40 % in man and 20 % in the dog and in the rat. The binding capacity is not exceeded at the plasma riboflavin levels obtained even after large doses. However, since the degree of plasma protein binding has not been established at physiological plasma riboflavin levels, a more extensive, possibly saturable binding to other proteins cannot be excluded. Such a binding would be of considerable importance for the physiological retention of riboflavin. Chickens exhibit unique binding characteristics because of the presence of a specific carrier protein in their plasma. The binding of riboflavin to this protein approximates 100 % and is of vital importance for the deposition of the vitamin in developing eggs.

The apparent *volume of distribution* depends on the method of determination and may be of questionable significance because of the complex pharmacokinetics involved. In the rat, estimates generally exceed the body weight, probably reflecting the extensive secretion of the vitamin into the intestine. In the dog and in the man, estimates of the apparent volume of distribution, based on a single compartment open model and first order elimination processes approximate 30 % of the body weight. Careful studies in both species have however, suggested more complex distribution kinetics, involving a smaller rapidly equilibrated compartment, representing 16 % of the body weight, as well as a larger, slowly equilibrated compartment. Such data *might* be interpreted as a rapid distribution in the extracellular space followed by a slow migration into the cells. The fact that the total distribution volume does not exceed the total body water volume, indicates that administered riboflavin is not extensively bound in tissues. This is consistant with the fact that the flavin binding capacity is saturable, and that maximum tissue levels are present when the supply is adequate. A more extensive binding of administered riboflavin might, on the other hand, be expected in individuals with submaximum flavin levels in the tissue. Thus, riboflavin deficient patients are known to retain a greater proportion of a test dose than do normal subjcets.

Following acute administration to rats the kidney, the liver and the intestine momentarily exhibit higher flavin levels than do the other tissues. This particular distribution pattern may be related to secretory mechanisms located in these organs i.e. tubular, biliary and intestinal secretion. The transport across the blood brain barrier has not been investigated, but in the rat, radioactive vitamin is taken up at equal rates by the retina and the liver. Riboflavin seems to be transported actively from the maternal to the foetal blood by the placenta.

Riboflavin is *eliminated* by excretion in the urine and faeces and by

in turn are bound to plasma and tissue proteins, and secretion into the intestine followed by *reabsorption*. Other processes remove the vitamin irreversibly from the body, thereby contributing to its *elimination*: excretion in the urine and faeces, and degradation.

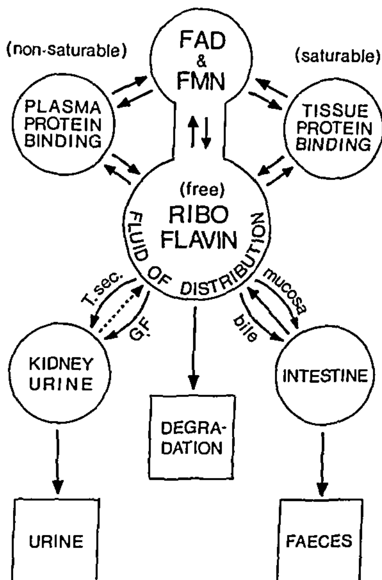


Fig 9 Schematic representation of the fate of riboflavin in the mammalian body Free riboflavin in the distribution fluids exists in dynamic equilibrium with flavins bound to proteins or located in various compartments as indicated by circles Elimination processes are indicated by irreversible arrows and squares Note the apparent similarity between the bidirectional transports of the vitamin between plasma and kidney urine and plasma and intestinal contents The phosphorylated forms of the vitamin (FMN and FAD) are probably not eliminated *per se* but only after hydrolysis to free riboflavin. GF = glomerular filtration, T sec = renal tubular secretion The broken arrow indicate that the renal reabsorption is easily saturable.

The binding of administered riboflavin to *plasma proteins* is not very extensive, and has possibly minor pharmacokinetic implications The fraction bound in the plasma, mainly to the albumin, approximates 40 % in man and 20 % in the dog and in the rat The binding capacity is not exceeded at the plasma riboflavin levels obtained even after large doses However, since the degree of plasma protein binding has not been established at physiological plasma riboflavin levels, a more extensive, possibly saturable binding to other proteins cannot be excluded. Such a binding would be of considerable importance for the physiological retention of riboflavin Chickens exhibit *unique binding characteristics* because of the presence of a specific carrier protein in their plasma The binding of riboflavin to this protein approximates 100 % and is of vital importance for the deposition of the vitamin in developing eggs

The apparent *volume of distribution* depends on the method of determination, and may be of questionable significance because of the complex pharmacokinetics involved In the rat, estimates generally exceed the body weight, probably reflecting the extensive secretion of the vitamin into the intestine In the dog and in the man, estimates of the apparent volume of distribution, based on a single compartment open model and first order elimination processes, approximate 30 % of the body weight. Careful studies in both species have, however, suggested more complex distribution kinetics, involving a smaller, rapidly equilibrated compartment, representing 16 % of the body weight, as well as a larger, slowly equilibrated compartment. Such data might be interpreted as a rapid distribution in the extracellular space followed by a slow migration into the cells The fact that the total distribution volume does not exceed the total body water volume, indicates that administered riboflavin is not extensively bound in tissues This is consistant with the fact that the flavin binding capacity is saturable, and that maximum tissue levels are present when the supply is adequate A more extensive binding of administered riboflavin might, on the other hand, be expected in individuals with submaximum flavin levels in the tissue Thus, riboflavin deficient patients are known to retain a greater proportion of a test dose than do normal subjects

Following acute administration to rats the kidney, the liver and the intestine momentarily exhibit higher flavin levels than do the other tissues This particular distribution pattern may be related to *secretory mechanisms* located in these organs, i e tubular, biliary and intestinal secretion. The transport across the blood brain barrier has not been investigated, but in the rat, radioactive vitamin is taken up at equal rates by the retina and the liver Riboflavin seems to be transported actively from the maternal to the foetal blood by the placenta.

Riboflavin is *eliminated* by excretion in the urine and faeces and by

degradation (designated by the squares in the model) The relative contribution of these processes depends on the *species* and the mode of administration Following parenteral administration, *renal excretion* predominates, accounting for up to 90 % of the dose in man, and more than 50 % in the dog and in the rat In addition to glomerular filtration, renal excretion involves active tubular secretion and probably saturable tubular reabsorption. There is no evidence of tubular reabsorption by passive diffusion The tubular secretion is non-saturable at plasma riboflavin concentrations attainable *in vivo* Probenecid inhibits the secretion The tubular reabsorption is easily saturable, and is thus without any quantitative significance at plasma levels exceeding the physiological level The renal handling of *administered* riboflavin therefore follows apparently first order kinetics during the greater part of the elimination The net clearance depends on the plasma riboflavin concentration, and is greater than the GFR at fairly high levels and less than the GFR at physiological levels

Faecal excretion of parenterally administered vitamin occurs in the rat, but probably not in man Riboflavin is secreted into the intestine by biliary as well as by mucosal secretion However, since the secreted vitamin may be reabsorbed, these processes do not necessarily result in the elimination of the vitamin On the contrary, intestinal secretion followed by reabsorption may prolong the availability of administered riboflavin It is uncertain whether species differences with regard to faecal excretion depend on differences in intestinal secretion or intestinal reabsorption There is evidence of biliar secretion in man, but secreted vitamin appears to be completely reabsorbed from the intestine In the rat, intestinal secretion occurs by way of the bile as well as directly via the mucosal cells In this species, as much as 20 % of a parenteral dose of riboflavin may be eliminated via the faeces

Elimination by *metabolic degradation* processes is well established in the rat A variable proportion (20–50 %) of orally administered riboflavin cannot be accounted for in the urine, faeces and carcass, and large quantities of radioactive metabolites have been detected in the urine and faeces following the administration of ^{14}C-labelled riboflavin One of these, lumichrome, is presumably produced by the intestinal microflora, while four unidentified non-isoalloxazine metabolites are thought to be produced in the tissues, mainly in the liver The extent to which riboflavin undergoes degradation in other mammals is not known Small amounts of isoalloxazine products resulting from side chain degradation of the riboflavin molecule have been detected in urine of humans and ruminants after large oral doses of riboflavin, but they are probably produced by intestinal microorganisms In man a possible formation of non-isoalloxazine metabolites has not been satisfactorily ruled out, and the fate of the vitamin not accounted for in the urine remains uncertain

Summary

Introduction

The present survey deals with the biological fate of riboflavin (vitamin B_2) in the mammalian body, i.e. its absorption, distribution, metabolism and excretion. The survey is based in part on the literature, and in part on my own investigations in the rat.

Chapter 1 Absorption

The intestinal absorption occurs rapidly in *man*, maximum plasma levels being reached within 1–2 hours. Although 50 % only is excreted in the urine, at least small doses are thought to be completely absorbed. The absorption is saturable, so that single doses exceeding 20 mg is not completely absorbed. Absorption takes place mainly in the upper small intestine. For this reason riboflavin is poorly absorbed from sustained release preparations. The presence of food or bile salts promotes absorption of saturation doses. On the basis of these data it is suggested that riboflavin is absorbed from the human intestine by a specialized mechanism.

As opposed to this, riboflavin is absorbed slowly along the entire intestinal tract *in the rat*, 5–10 % being excreted unchanged in the urine, while 10–20 % appears in the faeces indicating incomplete absorption. The absorption is not saturable. Riboflavin is transported slowly by preparations of intestine *in vitro*, and does not migrate against an electrochemical gradient. It is partly converted to FMN in the intestinal mucosa, but nothing is known as to the relationship between phosphorylation and absorption. These characteristics suggest that in the rat riboflavin is absorbed from the intestine by passive diffusion.

Under certain dietary conditions animals and man may utilize riboflavin produced by the intestinal microflora.

Absorption from parenteral sites has been little investigated, but it appears in general to be more rapid and effective than absorption following the oral route.

Chapter 2 Distribution

Contents of *endogenous riboflavin*, FMN and FAD in various tissues are

presented The total flavin content varies between 1 and 40 μg per g wet tissue, and decreases in the range liver, kidney, heart, intestine, brain, muscle, skin In general, FAD constitutes 80 %, FMN 18 % and riboflavin 2 % of the total content Well defined upper and lower limits exist for the flavin content of each tissue

Following injection in rats, transient accumulation occurs selectively in the kidney, liver and intestine The greatest accumulation is in the upper small intestine, probably reflecting the considerable secretory activity at this site The total volume of distribution of *administered* vitamin depends on the experimental conditions In the rat, it mostly exceeds the body weight, while in the dog and in man it accounts for approximately 30 % of the body weight The distribution space apparently includes various compartments, into which the vitamin migrates at different rates

Riboflavin is bound mainly to albumin in the blood plasma At fairly high plasma levels, the bound fraction approximates 40 % in man, and 20 % in the dog and in the rat A binding of this magnitude has presumably little influence on the retention of the vitamin It is, however, uncertain whether binding to other plasma proteins, e g globulins, may play a role at physiological plasma levels

Riboflavin seems to be transported actively from maternal to foetal blood in the placenta

Chapter 3 Metabolism

The metabolic changes involve synthetic processes, leading to the active coenzyme forms, and degradative processes, resulting in loss of vitamin activity

Enzymes catalyzing the synthesis of FMN and FAD, as well as enzymes causing their hydrolysis to riboflavin, are present in most and probably in all tissues Thus, all mammalian cells may be able to synthesize flavin coenzymes The coenzyme synthesis seems to be controlled by endocrine systems

Degradation of riboflavin is well known in bacteria, but among mammals it has been demonstrated only in rats The extent of degradation depends on the route of administration, there is thus maximum degradation after oral ingestion and minimum degradation after intravascular injection Four unidentified metabolites have been demonstrated in the urine and faeces of rats These metabolites do not have the fluorescence characteristic for flavins and they are more water soluble than riboflavin There is evidence that they are produced in the tissues, presumably in the liver, and not by bacterial degradation in the intestine There are no data on the possible degradation of riboflavin in other species

Chapter 4 Excretion

In most species excretion in the urine is the main route of elimination The extent of excretion is maximum following parenteral administration, and minimum following oral administration The opposite applies to the excretion in the faeces The renal excretion involves glomerular filtration, tubular secretion and tubular reabsorption The tubular secretion cannot be saturated at high plasma levels, but is inhibited by probenecid As opposed to this, tubular reabsorption is saturated at low plasma levels The renal clearance accordingly exceeds the GFR at fairly high plasma levels, but may be less than the GFR at physiological levels

In the rat, part of the dose is excreted in the faeces whether the vitamin is administered orally or parenterally Riboflavin is secreted into the intestinal cavity by way of the bile as well as directly from the mucosa. The extent of faecal excretion in other species is unknown In man, riboflavin secreted via the bile is, at least in part, reabsorbed from the intestine (enterohepatic circulation)

Milk contains appreciable amounts of riboflavin

Chapter 5 Conclusions and pharmacokinetic model

The present chapter summarizes the conclusions reached in the foregoing chapters, and on this basis a general model of the fate of riboflavin in mammals is presented

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Christensen S., Studies on riboflavin metabolism in the rat. IV Riboflavin elimination after exclusion of the portal circulation *Acta pharmacol et toxicol* 1969d, 27, 49-55

Christensen, S Renal excretion of riboflavin in the rat *Acta pharmacol et toxicol* 1971a, 29, 428-440

Christensen, S., Studies on riboflavin metabolism in the rat V Degradation of riboflavin 2 ^{14}C *Acta pharmacol et toxicol* 1971b, 30, 141-152

Chr. [illegible] metabolism in the rat. VI Properties of radio- [illegible]

Clagett, C. O Genetic control of the riboflavin carrier [illegible] 123-129

Clarke, H. C., The relationship between whole blood riboflavin levels and results of riboflavin saturation tests in normal and pathological conditions in man *Internat J Vit Res* 1969, 39 238-245

Cooperman, J M, H A Waisman, K. B McCall & C. A Elvehjem Studies on the requirements of the monkey for riboflavin and a new factor found in the liver *J Nutr* 1945, 30 45-57

Corduna, N A & I R Payne Absorption of riboflavin in chickens *Poultry Sci* 1967, 46, 1176-1179

Cornbleet, T., E R Kirch & O Bergeim Excretion of thiamine, riboflavin niacin and pantothenic acid in human sweat. *J A M A* 1943, June 12 426-429

Cowan, J W, R. V Boucher & E. G Buss Riboflavin utilization by a mutant strain of single comb white leghorn chickens 4 *Excretion and reabsorption of riboflavin* by the kidney *Poultry Sci* 1966, 45, 538-541

Dahlberg *et al* Sanitary milk control in relation to sanitary, nutritive and other *qualities of milk.* National Research Council Publ No 250, Washington 1952.

Daubenmerkl, W Undersøgelser over laktoflavinudskillelsen i urin Dissertation in Danish with an English summary Einar Munksgård, Copenhagen 1947

De H N & J K Roy *Effect of carbohydrates on the biosynthesis of riboflavin Indian* J Med Res 1951 39, 63-71

England, S Enzymatic phosphorylation of riboflavin to flavin mononucleotide in ani*mal tissues Fed Proc* 1952, 11 208-208

Everson, G E Wheeler H Walker & W J Caulfield Availability of riboflavin of ice cream peas and almonds judged by urinary excretion of the vitamin by women *subjects. J Nutr 1948* 35, 209-223

Faulkner R D & J P Lambooy Intestinal synthesis of riboflavin in the rat *J Nutr* 1961 75 373-378

Foley B R E MacKenzie & D B McCormick Transport and storage of ^{14}C-riboflavin in the retina and liver of rats *Proc Soc Exp Biol Med 1967*, 124 715-718.

Forster J W Microbiological aspects of riboflavin. I. *Introduction* (I Bacterial oxida*tion of riboflavin to lumichrome J Bacteriol* 1944, 47, 27-41

Fraser H F N H Topping & H Isbell The bacterial assay *of riboflavin in the urine* and tissues of normal and depleted dogs *and rats Pub Health Rep* 1940, 55, 280-289

Fridericia, L. S P Freudenthal S *Gudjonnsson, G* Johansen & N Schoubye Refection, a transmutable change *in the intestinal content, enabling* rats to grow and thrive without vitamin B in the food, *J Hygiene (London)* 1928, 27, 70-78

Goldstein A., L. Aronow & S. M Kalman. *Principles* of drug action, Harper & Row, Publishers, New York, Evanston, and London 1969

References.

Amos, W H, M Balaghi, O Ramirez & H E Sauberlich Metabolism of ^{14}C riboflavin in the rat *Fed Proc* 1966, **25**, 245–245

Axelrod, A E, T D Spies & C. A. Elvehjem A study of urinary riboflavin excretion in man *J Clin Invest* 1941, **20**, 229–232

Baker, H, O Frank, S Feingold & C M Leevy Vitamin distribution in human plasma proteins *Nature* 1967, **215**, 84–85

Berglund, F, A Engberg, E Persson & H Ulfendahl Renal clearances of labelled inulin (inulin carboxyl ^{14}C, inulin methoxy-^{3}H) and a polyethylene glycol (PEG 1000) in the rat *Acta Physiol Scand* 1969, **76**, 458–462

Bessey, O A, O H Lowry & R H Love The fluorometric measurement of the nucleotides of riboflavin and their concentration in tissues *J Biol Chem* 1949, **180**, 755–762

Bessey, O A, O H Lowry, E B Davis & J L Dorn The riboflavin economy of the rat *J Nutrition* 1958, **64**, 185–202

Brewer, W, T Porter, R Ingalls & M A Ohlson The urinary excretion of riboflavin by college women *J Nutr* 1946, **32**, 583–596

Bro Rasmussen, F The riboflavin requirement of animals and man and associated metabolic relations I Technique of estimating requirement, and modifying circumstances *Nutr Abstr Rev* 1958, **28**, 1–23

Brzezinski, A, Y M Bromberg & F G Sulman Prolonged effect of riboflavin Aluminium monostearate suspension in man *J Am Pharm Assoc* 1957, **46**, 109–111

Burch, H B, O A Bessey & O H Lowry Fluorometric measurements of riboflavin and its derivatives in small quantities of blood serum and cells *J Biol Chem* 1948, **175**, 457–470

Burch, H B, O H Lowry, A M Padilla & A M Combs Effects of riboflavin deficiency and realimentation on flavin enzymes of tissues *J Biol Chem* 1956, **223**, 29–45

Campbell, J A & A B Morrison Some factors affecting the absorption of vitamins. *Am J Clin Nutr* 1963, **12**, 162–169

Chen, C & K Yamauchi Absorption of riboflavin in the isolated intestine of rats. *J Vitaminol* 1950, **6**, 247–250

Chen, C & K Yamauchi Histochemical study on riboflavin *J Vitaminol* 1961, **7**, 163–181

Christensen, S Studies on riboflavin metabolism in the rat I Urinary and faecal excretion after oral administration of riboflavin 5 phosphate. *Acta pharmacol et toxicol* 1969a, **27**, 27–33

Christensen, S Studies on riboflavin metabolism in the rat II Metabolic flavin elimination after oral or intraperitoneal administration of riboflavin 5 phosphate *Acta pharmacol et toxicol* 1969b, **27**, 34–40

Christensen, S Studiees on riboflavin metabolism in the rat III Disappearance rate and urinary excretion rate of flavin after intravenous infusion of riboflavin 5 phosphate *Acta pharmacol et toxicol* 1969c, **27**, 41–48

Kornberg, A. & W E. Pricer Nucleotide pyrophosphatase. *J Biol Chem* 1950, 182, 763–778

Kuhn R., K Reinemund, H Kaltschmitt, R Strobele & H. Trischmann Synthetisches 6,7 Dimethyl 9-d Riboflavin. *Die Naturwissenschaften* 1935, 23, 260–260

Kunz, A F Der Lactoflavinstoffwechsel in zwei Fallen von generalisiertem Ekzem bei vorwiegender Behandlung mit Rohkost. *Schweiz Med Wchnschr* 1942, 23, 1154–1158

Lambooy, J P Riboflavin antagonists. *Bibl Nutritio et Dieta* 1966, 8, 139–155

Lane, M., J L. Fahey, R D Sullivan & C. G Zubrod The comparative pharmacology in man and the rat of the riboflavin analogue 6,7 dimethyl 9 (2 acetoxyethyl)-isoalloxazine, U 2112, *J Pharmacol Exp Ther* 1958, 122, 315–326

Laszt, L. & L. Dalla Torre Untersuchungen über die Laktoflavinausscheidung bei normalen und nebennierenlosen Tieren unter verschiedenen Bedingungen *Z Vitaminforsch* 1943, 13, 77–101

Leviton, A. & M J Palansch Binding of riboflavin and riboflavin phosphate by the proteins in milk *J Dairy Sci* 1960, 43, 1713–1724

Levy G & W J Jusko Factors affecting the absorption of riboflavin in man *J Pharm Sci* 1966a, 55, 285–289

Levy, G & W J Jusko Apparent renal tubular secretion of riboflavin in man. *J Pharm Sci* 1966b, 55, 1322–1322

Lossy, F T., G A Goldsmith & H P Sarett A study of test dose excretion of five B complex vitamins in man *J Nutr* 1951, 45, 213–224

Luckey T D, J R. Pleasants & J A Reyniers Germfree chicken nutrition II Vitamin interrelationships *J Nutr* 1955, 55, 105–118

Lust, J E, D D Hagerman & C A. Villee The transport of riboflavin by human placenta *J Clin Invest* 1954, 33 38–40

Macy, I. G Composition of human colostrum and milk. *Am J Dis Child* 1949, 78, 589–603

Mattews, D M Absorption of water soluble vitamins *Brit Med Bull* 1967, 23, 258–262.

Mayersohn, M & M Gibaldi Drug transport. II The effect of various cations on the passive transfer of drugs across the everted rat intestine *Biochem Biophys Acta* 1970, 196, 296–304

Mayersohn, M, S. Feldman & M Gibaldi Bile salt enhancement of riboflavin and flavin mononucleotide absorption in man *J Nutr* 1969, 98, 288–296

McCormick D B Flavokinase activity of rat tissue and masking effect of phosphatases *Proc Soc Exp Biol Med* 1961, 107, 784–786

McCormick D B & M Russell Hydrolysis of flavin mononucleotide by acid phosphatases from animal tissues. *Comp Biochem Physiol* 1962, 5, 113–121

Melnick, D H. Field & W D Robinson Physiological availability of the vitamins. I The human bioassay technique *J Nutr* 1945, 30, 67–75

Menon I A & E Søgnen Amprolium and transport of thiamine in suspensions of intestinal cells *Acta Vet Scand* 1971, 12, 111–113

Middleton E J & H C Grice Vitamin absorption studies. IV Site of absorption of C14-riboflavin and S35-thiamine in the rat. *Canad J Biochem* 1964, 42, 353–358

Miles, H T & E. R Stadtman Isolation and characterization of 6,7-dimethyl 9-(2'-hydroxyethyl)-isoalloxazine as a bacterial fermentation product of riboflavin. *J Am Chem Soc* 1955 77, 5746–4747

Miyao M, H Matsuda, T Usui, H Tanaka, T Kochi & Y. Yamagata Studies on the

Golman, K Epithelceller isoleret fra rottetyndtarmens mucosa Special subject thesis 1970 University of Copenhagen, Dept Biochem

Haenel H H *Ruttloff* & H Ackermann Vitamin sparing action of difficultly resorbable carbohydrates *Biochem Ztschr* 1959, 331, 209–215

Harkness, D R, L Tsai & E R Stadtman Bacterial degradation of riboflavin V Stoichiometry of riboflavin degradation to oxamide and other products, oxidation of C14 labelled intermediates and isolation of the pseudomonad effecting these transformations *Arch Biochem Biophys* 1964, 108, 323–333

Hemmerich, P, C Veeger & H C S Wood Fortschritte in Chemie und Molekularbiologie der *Flavine und Flavoenzyme Angew Chemie* 1965, 77, 699–716

Hirt, A & K Wimmer Luminescenzmikroskopische Untersuchungen am lebenden Tier *Klin Wschr* 1940, 18, 123–140

Hobson P N, R Sumners, E C Owen, J C Spencer & D W West A study of rumen anaerobic bacteria in the presence of excess riboflavin *Proc Nutr Soc* 1969, 28, 53A–54A

Hotta K & I Ishigura Influence of diets on the decomposition of riboflavin by rat feces *Vitamins* 1955, 9, 335–337

Innami, S, T Kawachi & H Oizumi Decomposition of riboflavin in rat intestine *Japanese J Nutr* 1965, 23, 1–7

Johnson, R E Rate of urinary excretion of ascorbic, thiamine, riboflavin and n' methyl nicotinamide and the effects of diuresis, alkalosis, acidosis and ingestion of food *Fed Proc* 1946, 5, 139–140

Jusko, W J The absorption, distribution, metabolism, and elimination of riboflavin in man Ph D thesis from the State University of New York at Buffalo, 1970

Jusko, W J & G Levy Absorption, metabolism, and excretion of riboflavin 5' phosphate in man *J Pharm Sci* 1967a, 56, 58–62

Jusko, W J & G Levy Effect of probenecid on riboflavin absorption and excretion in man *J Pharm Sci* 1967b, 56, 1145–1149

Jusko W J & G Levy Plasma protein binding of riboflavin and riboflavin 5 phosphate in man *J Pharm Sci* 1969, 58, 58–62

Jusko, W J & G Levy Pharmacokinetic evidence for saturable renal tubular reabsorption of riboflavin *J Pharm Sci* 1970, 59, 765–772

Jusko, W J, G Levy, S J Yaffe & R Gorodischer Effect of probenecid on clearance of riboflavin in man *J Pharm Sci* 1970a, 59, 473–477

Jusko, W J, J R Leonards & G Levy Riboflavin distribution and elimination in two functionally anephric human subjects *J Pharm Sci* 1970b, 59, 566–567

Jusko, W J, B R Rennick & G Levy Renal excretion of riboflavin in the dog *Am J Physiol* 1970c, 218, 1046–1053

Jusko, W J, S J Yaffe & J E Allen Riboflavin absorption in children with biliary obstruction *Am J Dis Child* 1971, 121, 48–52

Kagaya, A Intestinal absorption of riboflavin in malnourished dogs *Vitamins* 1956 11, 313–316

Karrer, P, K Schopp & F Benz Synthesen von Flavinen IV *Helvetica Chimica Acta* 1935 18, 426–429

Kasper, H Die Resorption von Cocarboxylase, Riboflavin 5 phosphat Natrium und Vitamin A im Colon *Z Ernahrungswiss* 1965, 6, 54–61

Kearney, E B & S Englard The enzymatic phosphorylation of riboflavin *J Biol Chem* 1951, 193, 821–834

Klein, J R & H I Kohn The synthesis of flavin adenine dinucleotide from riboflavin by human blood cells *in vitro* and *in vivo* *J Biol Chem* 1940. 136 177–189

Rivlin, R. S Regulation of flavoprotein enzymes in hypothyroidism and in riboflavin deficiency Advances Enzyme Regulation 1970a 8 239–250

Rivlin [illegible] Med 1970b, 283, 463–472.

Rivlin [illegible]

Rudy H Enzymatische Phosphorylierung des Lactoflavins [illegible] 287

Ruy, Y W, E S Kim & C S Song Riboflavin and thiamine absorption Yonsei Med J 1968, 9 11–13

Sakai M & K Hamada On the riboflavin decomposing bacteria in human feces II Riboflavin decomposition by strain 14-3 Bitamin 1960, 21, 209–216

Scheline, R R Drug metabolism by intestinal microorganisms J Pharm Sci 1968, 57, 2021–2037

Schottek, W Untersuchungen uber die normale Transportgeschwindigkeit eines Kontrastbreies im Ratten und Mausedunndarm Z Ges Exp Med 1966 141, 17–20

Sebrell, W H., R. E. Butler, J G Wooley & H. Isbell Human riboflavin requirement estimated by urinary excretion of subjects on controlled intake Publ Health Rep 1941, 56 510–519

Selye H The role played by the gastrointestinal tract in the absorption and excretion of riboflavin. J Nutr 1943 25 137–142

Smith H. W The kidney Structure and function in health and disease Oxford University Press, New York 1951

Spencer R P & K. R Brody Biotin transport by small intestine of rat, hamster, and other species. Am J Physiol 1964 206, 653–657

Spencer R. P & N Zamcheck Intestinal absorption of riboflavin by rat and hamster Gastroenterology 1961, 40 794–797

Sperber I A new method for the study of renal tubular excretion in birds. Nature 1946 158 131–135

Stripp B Intestinal absorption of riboflavin in man Acta pharmacol et toxicol 1965, 22 353–362.

Sure, B & Z. W Ford Influence of increasing doses of thiamine and riboflavin on efficiency of their utilization. J Nutr 1943 26, 659–671

Tedeschi G G Eliminazione of lactoflavin phosphoric acid by way of the bile Bol Soc Ital Biol Sper 1955 30 663–665

Theorell H & A P Nygaard Kinetics and equilibria in flavoprotein systems, II The effects of pH anions and temperature on the dissociation and reassociation of the old yellow enzyme Acta Chem Scand 1954 8 1649–1658

Turner J B & D E Hughes The absorption of some B group vitamins by surviving rat intestine preparations Quart J Exptl Physiol 1962 47 107–123

Weiner I M Mechanisms of drug absorption and excretion Ann Rev Pharmacol 1967 7 39–56

West, D W & E C Owen The urinary excretion of metabolites of riboflavin by man. Br J Nutr 1969 23, 889–898

Wiegand R G J D Buddenhagen & C J Endicott Multiple dose excretion kinetics J Pharm Sci. 1963, 52, 268–273

Wilson, T H Intestinal absorption. W B Saunders, Philadelphia 1962.

Wilson, T H & G Wiseman Use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface J Physiol 1954 123 116–120

Winter, W P., E. G Buss C. O Clagett & R. V Boucher The nature of the biochem

intestinal absorption of thiamine, riboflavin and calcium in liver damage *Toku shima J Exp Med* 1967, 14, 1–12

Mizuhara, S Fatees of thiamine and *riboflavin in vivo J Japan Biochem Soc* 1950, 22, 11–15

Morrison, A B & J A *Campbell* Vitamin absorption studies I Factors influencing *the excretion of oral test doses of thiamine and* riboflavin by human subjects. *J Nutr 1960, 72, 435–440*

Muto, T Experimental *studies* on *intestinal* absorption of riboflavin I Absorption of riboflavin and its ester *forms from* the small intestine of rats *Acta Paediatrica Japonica* 1959, 63, 1419–1426

Najjar V A & L E *Holt* A riboflavin excretion test as a measure of riboflavin defici ency in man *Bull Johns Hopkins Hosp 1941, 69, 476–481.*

Najjar, V A, *G A Johns, G C* Medairy, G Feischmann & L. E Holt The biosyn thesis of riboflavin in man *J A M A 1944, 126, 357-358*

Nakamura, T., *N Nomoto, R* Yagi & N Oya Metabolic B complex deficiency due to the *administration* of glucocorticiod *J Vitaminol* 1970, 16, 89–98

Nieman, C & *A P Jansen Urinary excretion of riboflavin in normal, diuretic, and* alloxan diabetic rats *Internat Z Vitaminforsch* 1955, 25, 448–458

Nogami, H, *M* Hanano, *S* Awazu & T Iga Pharmacokinetic aspects of biliary ex *cretion* Dose dependency of riboflavin in rat *Chem Pharm Bull* 1970, 18, 228–234

Okuda, J Metabolism of flavin nucleotides I Decomposition of flavin nucleotides in digestive juice *Chem Pharm Bull* 1958a, 6, 662–665

Okuda, J Metabolism of flavin nucleotides II Decomposition of flavin nucleotides in the small intestine Chem Pharm Bull 1958b, 6, 665–669

Okuda, J Metabolism of flavin nucleotides III Distribution of enzymes in the cells of the mucosa of small intestine causing dephosphorylation of flavin mononucleo tide *Chem Pharm Bull 1959, 7, 295–296*

Olcese, O & P B Pearson The destruction in the gastrointestinal tract of orally in gested riboflavin and pantothenic acid *Internat Z Vitalinforsch* 1948, 20, 128–133

Onishi, N Effect of ursodesoxycholic acid and adenosine on the intestinal absorption of riboflavin *Vitamins* 1956, 11, 479–481

Owen, E C & D W West Metabolites of riboflavin in milk, urine, and tissues of animals in relation to alimentary symbiotic bacteria *Br J Nutr* 1970, 24, 45–60

Pearson, P B, M K Sheybani & H Schmidt Riboflavin in the nutrition of the horse *Arch Biochem Biophys* 1944, 3, 467–474

Phillips, J W Physical and chemical properties of a riboflavin binding protein Ph D *thesis, Pennsylvania State University, 1969*

Ponz, F Der Einfluss von Thyroxin auf die Resorption und Ausscheidung von Lacto flavin *Z Vitaminforsch* 1944, 14, 213–230

Potter, R L., A E Axelrod & C A Elvehjem The riboflavin requirement of the dog *J Nutr* 1942, 24, 449–460

de Preux, R Die Rolle der Leber und der Nieren im Lactoflavinstoffwechsel *Z Ges Expil Med* 1942, 110 12–22

Pulver, R & F Verzar Die Phosphorylierung von Riboflavin durch Darmschleimhaut. *Enzymologia* 1939, 6, 333–336

Rama Sastri, B V, R Mukundan & V N Patwardhan Urinary excretion of riboflavin and thiamine in Indian adults *Indian J Med Res* 1950, 38, 213–233

Rennick, B R Renal tubular *excretion of riboflavin in* the chicken Proc Soc Expil Biol Med 1960, 103, 241–243

Reviews.

Arvy, L. Le rein et la riboflavine *Laval Med* 1969, 40, 383–394

Blum, J C. Les formes de transport des vitaminees hydrosolubles. *Ann. Nutr. Alim* 1969, 23, A5–A25

Bro-Rasmussen, F The riboflavin requirement of animals and man and associated metabolic relations. I Technique of estimating requirement, and modifying circumstances. *Nutr Abstr Rev* 1958, *28*, 1–23

Bro-Rasmussen F The riboflavin requirement of animals and man and associated metabolic relations. II The relation of requirement to the metabolism of protein and energy *Nutr Abstr Rev* 1958, 28, 369–386

Hemmerich, P., C Veeger & H C. S. Wood Fortschritte in Chemie und Molecular biologie der Flavine und Flavoenzyme *Angew Chemie* 1965, 77, 699–716

Jusko, W J The absorption, distribution, metabolism, and elimination of riboflavin in man. Ph. D thesis State Uuniversity of New York at Buffalo, 1970

Lambooy J P., Riboflavin antagonists *Bibl Nutritio et Dieta* 1966, 8, 139–155

Neims, A H & L. Hellerman. Flavoenzyme catalysis *Ann Rev Biochem* 1970, 39, 867–887

Rivlin, R. S. Riboflavin metabolism *New Engl. J Med* 1970, 283, 463–472

Stadtman, E. R The biosynthesis and degradation of riboflavin *Proc 4th Biochem Congr*, Vienna (1958) 1960, 11, 19–31

Shaw, J H & P H Philips The pathology of riboflavin deficiency in the rat. *J Nutr* 1954, 22, 345–358

Yagi K Flavins and flavoproteins. University Park Press, Baltimore 1969

ical lesion in avian renal riboflavinuria II The inherited change of a riboflavin binding protein from the blood and eggs *Comp Biochem Physiol* 1967, **22**, 897–906

Wiseman, G Absorption from the intestine Academic Press, London 1964

Wood, H O The surface area of the intestinal mucosa in the rat and in the cat *J Anat* 1944, **78**, 103–105

Yagi, K Distribution of riboflavin nucleotides in rat organs influenced by the administration of flavin compounds *J Biochem* 1954a, **41**, 757–762.

Yagi, K Destination of injected vitamin B_2 II Emigration of vitamin B_2 to intestinal chanal through intestinal mucosa *Vitamins* 1954b, **7**, 36–37

Yagi, K & J Okuda Phosphorylation of riboflavin by transferase action *Nature* 1958, **181**, 1663–1664

Yagi, K, T Nagatsu, I Nagatsu Ishibashi & A Ohashi Migration of injected C^{14}-labelled riboflavin into rat tissues *J Biochem* 1966, **59**, 313–315

Yagi, K, M Yamada & J Okuda Studies on fatty acid esters of flavins XVI Excretion of riboflavin and riboflavin tetrabutyrate into bile after injection of C^{14} labelled riboflavin tetrabutyrate *Vitamins* 1968, **37**, 289–293

Yamagiri, K Intracellular distribution of flavin containing enzymes in the liver of normal and riboflavin deficient rats I Distribution of FAD and FMN *Vitamins* 1958 **14**, 765–769

Yanagita, T & J W Forster A bacterial riboflavin hydroxylase. *J Biol Chem* 1956, **221**, 593–607

Yang, C. & D B McCormick Degradation and excretion of riboflavin in the rat *J Nutr* 1967, **93**, 445–453

Yoshihara, S Studies on the mechanism of riboflavin absorption I Distribution and absorption of riboflavin in digestive tracts of normal rabbits *Vitamins* 1954, **7**, 763–767

Reviews

Arvy, L. Le rein et la riboflavine. *Laval Med* 1969, 40, 383–394
Blum, J C. Les formes de transport des vitaminees hydrosolubles *Ann Nutr Alim* 1969, 23, A5–A25
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Kapitel 2 Fordeling

Indledningsvis gives en oversigt over det *endogene* flavinindhold i forskellige væv Det totale flavinindhold varierer mellem 1 og 40 µg per g vådt væv, aftagende i rækkefølgen lever, nyre, hjerte, tarm, hjerne, muskler og hud. I almindelighed udgør flavin adenin dinukleotid (FAD) 80 %, flavin mononukleotid (FMN) 18 % og riboflavin 2 % af totalindholdet Der eksisterer veldefinerede øvre og nedre grænser for flavinindholdet i de enkelte væv

Efter injektion af riboflavin eller FMN ses hos rotter en kortvarig, selektiv akkumulation i nyre, lever og tarm Akkumulationen er betydeligst i duodenum, hvor den afspejler sekretion af vitaminet ud i tarmkanalen Det tilsyneladende fordelingsvolumen for *administreret* riboflavin afhænger af forsøgsomstændighederne Hos rotter oversiger det hyppigt kropsvægten, medens det hos hunde og mennesker andrager ca 30 % af denne Fordelingsrummet omfatter flere "compartments", hvortil vitaminet fordeler sig med forskellig hastighed

Riboflavin og flavin mononukleotid bindes til blodplasmaets albumin. Bindingsgraden er ved højere plasmakoncentrationer konstant og andrager for riboflavin ca. 40 % hos menneesker og ca 20 % hos hunde og rotter En binding af denne størrelsesorden har næppe større betydning for organismens retention af vitaminet Det er imidlertid ikke klarlagt om eventuelle bindinger til andre plasmaproteiner finder sted ved fysiologiske plasmakoncentrationer

Riboflavin transporteres tilsyneladende aktivt fra moder- til fosterblod i placenta.

Kapitel 3 Metabolisering

Der skelnes mellem syntetiske processer, hvorved riboflavin omdannes til dets biologisk aktive former, og degraderingsprocesser, hvorved vitaminets aktivitet går tabt.

De fleste væv indeholder enzymer, som katalyserer biosyntesen af FMN og FAD samt disses hydrolyse til riboflavin Det er sandsynligt, at alle organismens celler er i stand til at producere FMN og FAD ud fra riboflavin Syntesen af flavin koenzymer er formentlig under hormonal kontrol

Biologisk omdannelse af riboflavin til produkter uden vitaminvirkning er velkendt hos visse mikroorganismer, men hos pattedyr hidtil kun påvist i betydeligt omfang hos rotter Metaboliseringsintensiteten afhænger hos denne dyreart af administrationsmåden og er betydeligst efter peroral indgift (indtil 25 % af dosis) og mindst efter injektion i blodbanen Der er påvist 4 uidentificerede riboflavinmetabolitter i urin og fæces fra rotter Disse mangler den karakteristiske flavin fluorescens og er mere hydrofile end riboflavin Det er

Resume

Indledning

Den foreliggende oversigt omhandler riboflavin (vitamin B_2)s skæbne i pattedyrsorganismen, specielt forholdene vedrørende vitaminets absorption, fordeling, metabolisering og udskillelse Oversigten er baseret dels på litteraturstudier, dels på originale undersøgelser hos rotter

Kapitel 1 Absorption

Hos *mennesker* sker absorptionen fra mave-tarmkanalen hurtigt, maksimale plasmakoncentrationer opnås allerede efter 1–2 timer Selv om kun ca halvdelen af en peroral dosis genfindes i urinen, antages det, at mindre enkeltdoser absorberes fuldstændigt Absorptionen er saturabel, således at enkeltdoser større end ca 20 mg absorberes mindre effektivt Optagelsen finder overvejende sted i den proximale del af tyndtarmen, hvorfor absorptionen er mindre effektiv fra "sustained release" præparater Tilstedeværelse af føde eller galdesalte fremmer absorptionen af satureringsdoser Det antages, bl a på grundlag af disse forhold, at riboflavin hos mennesker optages fra tarmen ved en "speciel" absorptionsmekanisme, ligesom det er tilfældet med vitamin B_1 og B_{12}

Tarmabsorptionen hos *rotter* er til sammenligning en relativ langsom proces, som finder sted ned igennem hele tarmkanalen Kun 5–10 % udskilles uomdannet med urinen, medens 10–20 % udskilles med fæces som tegn på ufuldstændig absorption Optagelsen kan ikke mættes Riboflavin transporteres langsomt af tarmpræparationer *in vitro* og kan ikke optages imod en elektrokemisk gradient Det omdannes delvist til flavin mononukleotid i tarmslimhinden, men relationen mellem absorption og fosforylering er ukendt De anførte forhold taler for, at riboflavin absorberes fra rottetarmen ved simpel diffusion

Både mennesker og dyr er under visse ernæringsmæssige forhold i stand til at udnytte riboflavin, der er produceret af mikroorganismer i mave-tarmkanalen

Absorptionen efter parenteral administration er lidet undersøgt, men synes, i det mindste hos rotter, at være hurtigere og mere effektiv end efter peroral indgift

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sandsynliggjort at disse metabolitter skyldes nedbrydning i vævene, fortrinsvis i leveren, og ikke bakteriel nedbrydning i tarmkanalen Mindre mængder af den fluorescerende metabolit lumikrom produceres antageligt af tarmfloraen Det er ikke klarlagt, i hvilket omfang riboflavin metaboliseres hos andre pattedyr

Kapitel 4 Udskillelse

Udskillelsen med urinen er den vigtigste eliminationsmåde hos de fleste arter Hos rotter er udskillelsesfraktionen større efter parenteral end efter peroral administration Det omvendte er tilfældet for den fækale udskillelse

Den renale udskillelse omfatter glomerulær filtration, tubulær sekretion og tubulær reabsorption Den tubulære sekretion blokeres af probenecid, men kan ikke mættes inden for opnåelige plasmakoncentrationer Derimod mættes reabsorptionen allerede ved plasmakoncentrationer i det fysiologiske område Den renale clearance overstiger derfor den glomerulære filtrationshastighed ved alle nogenlunde høje plasmakoncentrationer, medens den ved lavere plasmakoncentrationer kan antage værdier mindre end den glomerulære filtrationshastighed

Hos rotter udskilles en væsentlig del af dosis med fæces, hvadenten riboflavin administreres peroralt eller parenteralt Sekretionen af riboflavin ud i tarmlumen sker dels med galden, dels direkte fra tarmslimhinden Omfanget af den fækale udskillelse hos andre dyrearter er ikke undersøgt Hos mennesker reabsorberes secerneret vitamin delvist fra tarmen (enterohepatisk kredsløb)

Modermælk indeholder betragtelige mængder riboflavin

Kapitel 5 Konklusioner og farmakokinetisk model

I dette kapitel sammenfattes konklusionerne fra de foregående kapitler, og på grundlag heraf opstilles en almen model for riboflavins skæbne i pattedyrsorganismen

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I Biliary excretion of penicillins in the rat
  J Pharm Pharmac 1971, 23, 463—464

II Biliary excretion of ampicillin, azidocillin and benzylpenicillin in rat
  Acta pharmacol et toxicol 1973
  (Together with N O Bodin and E Hansson) (in press)

III Uptake and degradation of ^{3}H phenoxymethylpenicillin in rat liver slices
  Acta Pharm Suecia 1973 (in press)

IV Biliary excretion of quaternary ammonium compounds and tertiary amines in the rat
  Acta pharmacol et toxicol 1971, 30, 59—68
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These papers will be referred to in the text by their Roman numerals

ABBREVIATIONS

ATP	adenosine triphosphate
BSP	sulphobromophthalein
DNP	2,4-dinitrophenol
IAA	iodoacetic acid
PAEB	procaineamide ethobromide
PcV	phenoxymethylpenicillin
Mw	*molecular weight*
S/M	slice (100 mg)-to medium (100 μl) concentration ratio

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INTRODUCTION

Biliary excretion of endogenous and exogenous compounds is the most important elimination pathway besides elimination in the urine Knowledge concerning the biliary excretion of compounds is of importance from the pharmacological and toxicological point of view A high exposure of an antibiotic agent to the hepato-biliary tract is adventageous if an infection is confined to this area of the body (Coppi et al , 1971) From the diagnostic point of view it is important to find suitable biliary radiocontrast agents (Warrick, 1965) Extensive biliary excretion may be deleterious by exposing the hepato-biliary intestinal tract to high concentration of the substance if this is toxic e g intestinal cancer by 4-aminodiphenyl (Walpole et al , 1952) The possibility of drug accumulation prevails on repeated administration, if entero-hepatic circulation occurs e g glutethimide in man (Bakke, 1970)

In spite of extensive research, the formation of bile is incompletely understood Many reviews have been published concerning bile formation and secretion by e g Sperber (1959, 1963 & 1965), Wheeler (1968) and Schanker (1968) There is considerable evidence that formation of a primary bile is a process confined to the parenchymal cells of the liver lobulae This bile is, at least partly, formed by a transport of anions, primarily those of bile acids, from the sinusoids (blood conducting system) to the bile canaliculi (bile conducting system) via the liver parenchyma This event leads to a flow of water and solutes, which is caused by osmotic filtration and diffusion, into and down the biliary tree This theory was originally proposed by Sperber (1959) More recent investigations indicate that a portion of canalicular bile production is bile salt independent (Wheeler et al 1968) During the passage down the biliary ductules and ducts the primary bile is very probably modified by absorption and/or secretory process (Wheeler 1968) In some species, a secretin dependent bile fraction (HCO_3^- rich bile salt free) seems to be related to the biliary ductular cells (Wheeler & Ramos, 1960 and Forker, 1967) The mechanisms by which many compounds enter the bile may be closely associated with mechanisms for bile formation

Brauer (1959) has classified compounds which appear in the bile according to their bile to blood concentration ratios Group A represents those agents showing about the same concentration in bile and blood e g Na^+, K^+ and Cl^- and glucose Group B includes those compounds showing a higher concentration in the bile than in the blood e g bile acids and bilirubin and Group C encompasses compounds showing a lower concentration in the bile than in the blood e g proteins.

METHODS

The methods used are discribed in detail in the separate studies and are only briefly summarized here

In this investigation some of the penicillins used were available as radioactively labelled compounds and all of the quaternary ammonium compounds and tertiary amines were radioactively labelled

To study the excretion of the compounds into the bile, bile fistula rats of the Sprague Dawley strain were used The compounds were always injected into the femoral vein The animals were kept under pentobarbitone anesthesia and with a temperature of 38 $\pm 1^{\circ}$C, during the experiment (I, II and IV) In some experiments the renal pedicles were ligated and the carotid artery cannulated (II)

Incubation studies with liver slices in Tyrode s buffer were used to study the uptake of compounds into the liver (II, III and V) The incubations were performed in carbogene atmosphere (93 5 % O_2 and 6 5 % CO_2) and at 37°C The effects of metabolic inhibitors, structural analogues as well as other organic compounds on the uptake process were investigated (III and V) In some of the studies the influence of serum in the incubation medium on the uptake rate of compounds was investigated (II)

Ultrafiltration studies using liver homogenate were performed to investigate binding of compound to liver cell components (III and V)

To study the binding of penicillins to liver cytosol components, ultracentrifugation (100 000 g for 2 hours) was performed followed by gel-filtration (Sephadex G 75) Fractions were collected and assayed for anions and protein (absorbance at $\lambda = 280$ nm) (V)

Microbiological assay (I and II) liqu d scintillation counting (II—V), spectrophotometry (I and V) and chromatography (II—V) were used as analytical tools

Physicochemical properties of organic compounds such as polarity, molecular size and structural features are said to be of importance for their biliary excretion (e g Sperber, 1963, Williams et al , 1965, Schanker, 1965 and Millburn et al , 1967) It is suggested that compounds with molecular weights larger than about 300 and with anionic and/or cationic groups are prone to biliary excretion Reviews concerning biliary elimination of various organic compounds have been given by e g Smith (1966 & 1971) and Schanker (1968)

The transfer of organic compounds from blood to bile seems to be performed by at least two different (probably more) transport systems Organic cations, anions and possibly cardiac glucosides are said to be excreted by separate transport systems in the liver (Schanker, 1968) The transfer from blood to bile via the liver parenchyma may be the consequence of several simultaneous processes e g simple diffusion, facilitated diffusion and energy dependent transfer at both sinusoidal and canalicular sites Incubation studies with liver slices indicate that the uptake of organic cations is at least partly energy dependent (Solomon & Schanker, 1963, Gigon et al , 1969 and Broen-Christensen, 1970) Contributing to the uptake may also be tissue binding (Gigon et al , 1969) In studies with organic anions, no clear evidence of energy mediated uptake into liver slices has been obtained (Brauer & Pessotti, 1949, Barber-Riley, 1962 and Hargraves & Price, 1966) It has been suggested that binding to liver parenchyma components may be an important step in the excretion procedure of e g sulphobromophthalein (BSP) and bilirubin (Cornelius et al , 1967, Lewi et al , 1969 and Ockner et al , 1972)

The present investigation was performed to study the biliary excretion of some penicillins, quaternary ammonium compounds and tertiary amines The conjugate bases of penicillins can be taken as examples of organic anions and the quaternary ammonium compounds and conjugate acids of tertiary amines as exemples of organic cations Some physicochemical properties such as molecular weight, polarity and structural features of the compounds are discussed with respect to biliary excretion In these excretion studies bile fistula rats were used To clucidate transport characteristics of liver parenchyma for such compounds incubation studies with rat liver slices were performed

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To study the binding of penicillins to liver cytosol components, ultracentrifugation (100,000 g for 2 hours) was performed followed by gelfiltration (Sephadex G-75) Fractions were collected and assayed for anions and protein (absorbance at $\lambda = 280$ nm) (V)

Microbiological assay (I and II), liquid scintillation counting (II—V), spectrophotometry (I and V) and chromatography (II—V) were used as analytical tools

Physicochemical properties of organic compounds such as polarity, molecular size and structural features are said to be of importance for their biliary excretion (e g Sperber, 1963, Williams et al , 1965, Schanker, 1965 and Millburn et al , 1967) It is suggested that compounds with molecular weights larger than about 300 and with anionic and/or cationic groups are prone to biliary excretion Reviews concerning biliary elimination of various organic compounds have been given by e g Smith (1966 & 1971) and Schanker (1968)

The transfer of organic compounds from blood to bile seems to be performed by at least two different (probably more) transport systems Organic cations, anions and possibly cardiac glucosides are said to be excreted by separate transport systems in the liver (Schanker, 1968) The transfer from blood to bile via the liver parenchyma may be the consequence of several simultaneous processes e g simple diffusion, facilitated diffusion and energy dependent transfer at both sinusoidal and canalicular sites Incubation studies with liver slices indicate that the uptake of organic cations is at least partly energy dependent (Solomon & Schanker, 1963, Gigon et al , 1969 and Broen-Christensen, 1970) Contributing to the uptake may also be tissue binding (Gigon et al , 1969) In studies with organic anions, no clear evidence of energy mediated uptake into liver slices has been obtained (Brauer & Pessotti, 1949, Barber-Riley, 1962 and Hargraves & Price, 1966) It has been suggested that binding to liver parenchyma components may be an important step in the excretion procedure of e g sulphobromophthalein (BSP) and bilirubin (Cornelius et al , 1967, Lewi et al 1969 and Ockner et al , 1972)

The present investigation was performed to study the biliary excretion of some penicillins, quaternary ammonium compounds and tertiary amines The conjugate bases of penicillins can be taken as examples of organic anions and the quaternary ammonium compounds and conjugate acids of tertiary amines as exemples of organic cations Some physicochemical properties such as molecular weight, polarity and structural features of the compounds are discussed with respect to biliary excretion In these excretion studies bile fistula rats were used To elucidate transport characteristics of liver parenchyma for such compounds incubation studies with rat liver slices were performed

showed that the excretion was high (Table 1) The amount of active penicillin varied from about 10 % up to about 40 % of the given dose, during a four hour collection period Penicillins such as ampicillin, methicillin, and carboxybenzylpenicillin reached high bile concentrations The concentrations of active penicillin also persisted at high levels during the sampling period The cumulative excretion amounted from 33—38 % of the given dose Benzylpenicillin was also eliminated in appreciable amounts since about 25 % of the dose was found in the bile The other penicillins studied showed a more moderate excretion with a range from 11—19 % Table 1 shows, besides cumulative excretion, also the logarithm of the partition coefficient (log P), chromatographic R_M values $\left[R_M = \log\left(\frac{1}{R_F} - 1\right)\right]$ and molecular weights of the compounds (for definition of log P and R_M values, see Table 1) When relating the amount of biologically active penicillin excreted in the bile during the 0—4 hours period to log P values or R_M-values of the penicillins, correlation coefficients of —0 87 and —0 89 or —0 84 respectively, were found This indicates that with the penicillins used a relation exists between increasing polarity in the side chain of the penicillins and the amount of active compound found in the bile It may be possible that there will be no statistically proved correlation on changing the structure of the side chain drastically (e g as a result of steric factors) It was suggested that the variation in polarity may express differences in e g inactivation, affinity for transport systems and/or plasma protein bind ng of penicillins However, it must also be remembered that with the technique used the kidneys are functionally active and there is competition between excretion in the bile and urine

Paper II described biliary elimination of three ^{35}S-labelled penicillins, ampicillin, benzylpenicillin and azidocillin In the first study (I) ampicillin had shown extensive excretion, azidocillin low and benzylpenicillin was in between, these compounds were therefore suitable for studying the importance of e g inactivation and plasma protein binding for the amount of active penicillin eliminated in the bile The compounds were given intravenously (15 mg/kg) to bile fistula rats with ligated renal pedicles

The results showed that the excretion of total radioactivity was about the same for the three penicillins, 65—72 % of the given dose during the one hour collection period However, the amounts or biologically active penicillin excreted varied Ampicillin showed the highest excretion with about 40 % of the given dose in the bile while the corresponding values for benzylpenicillin and azidocillin were 19 % and 12 %, respectively These results are in agreement with the findings of the earlier investigation (I)

The observed difference in excretion of biologically active material indicates differences in inactivation of the penicillins As support for this,

RESULTS AND DISCUSSION

Penicillins

Biliary excretion of penicillins (I and II)

Much attention has been paid to the biliary excretion of organic compounds giving anions such as BSP (e.g. Brauer and Pessotti, 1949, Barber Riley, 1962 and Vanlerenberghe, 1970), and bilirubin (e.g. Brown et al., 1964 and Bernstein et al., 1966). Also, the biliary excretion of penicillins has been investigated (e.g. Stewart & Harrison, 1961, Ayliffe & Davis, 1965 Acred et al., 1961 and Henegar et al., 1961), but these studies have mostly been performed as an integral part of general metabolic studies of penicillins. Little work of a comparative nature with various penicillins has been made especially as regards molecular properties.

In paper (I) the biliary excretion of 10 penicillins was investigated after intravenous administration (15 mg/kg) in bile fistula rats. Since penicillins can give anions with molecular weights larger than about 300 extensive biliary excretion may be assured (>10 %) according to e.g. Sperber (1963), Williams et al. (1965) and Millburn et al. (1967). The results

Table 1 Biliary excretion of biologically active penicillin after intravenous administration of various penicillins (15 mg/kg) to rats. The cumulative excretion during 4 hours, expressed as % of the administered dose, is given together with log P, R_M-values and molecular weights of the penicillins.

Compound	Molecular weight	log P[a]/	log P[b]/	R_M[c]/	Amount excreted (%)[d]/
dicloxacillin	471	3.24	2.83	1.62	12.7 ±2.0
cloxacillin	437	2.49	2.44	1.34	11.2 ±1.9
oxacillin	401	2.38	2.34	1.05	18.9 ±2.6
azidocillin	375	2.29	—	—	12.9 ±1.9
pheneticillin	364	2.20	2.19	1.03	12.3 ±1.7
phenoxymethylpenicillin	350	2.03	2.01	0.89	15.1 ±1.8
benzylpenicillin	334	1.72	1.76	0.55	24.1 ±3.4
carboxybenzylpenicillin	378	1.13	—	—0.46	33.2 ±2.6
methicillin	380	1.06	1.13	0.47	38.4 ±5.1
ampicillin	349	—	—	0.07	33.2 ±4.8
correlation coefficient[e]/		—0.87	—0.89	—0.84	
significance		<0.001	<0.01	<0.01	

[a]/ log P values of free acids between n-octanol/acetate buffer 10 mmole/l pH 4.70 ±0.02 (Ryrfeldt (I))

[b]/ log P values of the free acids between n-octanol/water (Bird & Marshall 1971)

[c]/ R_M values determined by reserved phase thin layer chromatography acetone mobile phase (Biagi et al. 1969)

[d]/ each value is the mean of 3—7 experiments ±S.E.M.

[e]/ relating the amount of penicillin excreted in the bile to log P or R_M values

of about 4 was obtained after 60 minutes incubation However, a constant level of radioactivity attributable to unchanged drug was reached rapidly (10—15 min) with an S/M ratio of about 0 9 The metabolic inhibitors, iodoacetic acid (IAA) and 2 4-dinitrophenol (DNP) both caused a marked decrease in the uptake of total radioactivity but had different effects on the uptake of unchanged compound even at short incubation times (5—10 min) With IAA no decrease in uptake of radioactivity attributable to unchanged PcV could be detected, but significant reduction in uptake of total radioactivity was noted However, with DNP a parallel decrease in uptake of total radioactivity and activity attributable to unchanged PcV was obtained The ratio of unchanged to total radioactivity in this study was the same as that found in control experiments Generally, both IAA and DNP are considered to be metabolic inhibitors causing a decrease or stop of generation of ATP necessary for e g energy mediated transport The obtained results may be interpreted in the following way The effect of IAA is caused by inactivation of enzyme(s) responsible for the degradation of PcV These results with IAA gives no indication of an energy dependent uptake of PcV Preliminary incubation studies in a N_2-atmosphere gave no indication of energy dependent uptake of PcV (to be published)

The effect of DNP may be a competitive effect between PcV and DNP for tissue binding sites representing uptake and/or inactivation It can be said that the structural resemblance between DNP and PcV is sligth, so that competition for tissue binding sites should not occur However, both DNP and penicillin contain an aromatic structure and it is known that the side chain of penicillin which contains the aromatic structure is that part of the molecular which determines the extent of protein binding (Bird & Marshall 1967) It is known that simple aromatic substances are able to displace larger and more complicated substances containing aromatic structures from protein binding sites (Krieglstein et al 1972) The results with DNP could also be interpreted as the consequence of the inhibition of an energy (e g ATP) dependent uptake but this is in contradistinction to the interpretation of the effect of IAA If the uptake was energy dependent, IAA would very probably exert inhibition on the uptake of unchanged drug, since IAA should decrease stores and generation of ATP The preliminary incubation studies in N_2-atmosphere also indicate the absence of an energy dependent uptake of PcV Investigations with other anions support this view (Barber Riley 1962 Brauer & Pessotti 1949 and Hargreaves & Price, 1966) However these investigations using the liver slice technique, do not exclude the possibility of involvement of energy in some step(s) in the transfer of organic anions from blood to bile

Other compounds which caused inhibition of the uptake of PcV were organic anions such as those of BSP and probenecid The effect of these

the result from the incubation studies with liver slices can be taken These results showed that ampicillin was more stable to inactivation than benzylpenicillin, which was more stable than azidocillin In studies with perfused rat liver, Kind et al (1968) showed that ampicillin was more stable to inactivation than benzylpenicillin Preliminary incubation studies in rat liver homogenate have shown that ampicillin is more stable to degradation than phenoxymethylpenicillin and azidocillin (to be published) Since Bird and Marshall (1967) have demonstrated a correlation between lipophilicity, related to the side chain of the penicillins, and protein binding (high lipophilicity — high protein binding) the results here may suggest that azidocillin has a higher affinity for the enzyme(s) responsible for the degradation than benzylpenicillin and ampicillin However, factors such as steric properties may complicate this hypothesis It may also be possible that the higher lipophilicity of some of the penicillins may permit them to diffuse back from the primary bile, with a subsequent longer exposure time to enzyme(s) responsible for degradation of penicillins

Chromatographic studies indicated that one main metabolite was formed which probably is the penicilloic acid of the corresponding penicillin Small amounts and traces of other metabolites were also formed, but the chemical structures of these metabolites were not investigated here

Concerning the influence of plasma protein binding on excretion, it is imaginable that binding to plasma proteins can lead to a retarded excretion rate The uptake rate of azidocillin, which shows high protein binding, into liver slices was reduced when serum was present in the medium compared with control experiments (II) The influence of protein binding on the route of excretion (biliary-urinary) is an interesting aspect According to e g Baker & Bradley (1966) and Sperber & Sperber (1971) it is possible that renal excretion is more impeded by protein binding than biliary excretion, because of differences in anatomical arrangement between liver and kidney It has also been assumed that high affinity for components, e g protein in the membrane, and/or intracellularly of the liver parenchyma promotes biliary excretion according to e g Cornelius et al (1967), Lewi et al (1969) and Ockner et al (1972)

Transport studies with phenoxymethylpenicillin (III)

To elucidate transport characteristics of the liver parenchyma for penicillins, incubation studies with rat liver slices were performed under various metabolic conditions In this study tritium labelled phenoxymethylpenicillin (^{3}H-PcV), with high specific activity (71 μCi/mg) was used

The incubation studies showed that the uptake of total radioactivity was time and concentration dependent When the medium concentration of PcV was 10 μmole/l, an S/M-value (slice to-medium concentration ratio)

of about 4 was obtained after 60 minutes incubation However, a constant level of radioactivity attributable to unchanged drug was reached rapidly (10—15 min) with an S/M-ratio of about 0 9 The metabolic inhibitors, iodoacetic acid (IAA) and 2,4-dinitrophenol (DNP) both caused a marked decrease in the uptake of total radioactivity but had different effects on the uptake of unchanged compound even at short incubation times (5—10 min) With IAA no decrease in uptake of radioactivity attributable to unchanged PcV could be detected, but significant reduction in uptake of total radioactivity was noted However, with DNP a parallel decrease in uptake of total radioactivity and activity attributable to unchanged PcV was obtained The ratio of unchanged to total radioactivity in this study was the same as that found in control experiments Generally, both IAA and DNP are considered to be metabolic inhibitors causing a decrease or stop of generation of ATP necessary for e g energy mediated transport The obtained results may be interpreted in the following way The effect of IAA is caused by inactivation of enzyme(s) responsible for the degradation of PcV These results with IAA gives no indication of an energy dependent uptake of PcV Preliminary incubation studies in a N_2-atmosphere gave no indication of energy dependent uptake of PcV (to be published)

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Other compounds which caused inhibition of the uptake of PcV were organic anions such as those of BSP and probenecid. The effect of these

two compounds was the same as that found with DNP Also azidocillin could decrease the uptake, but no such effect was obtained with ampicillin It is known that azidocillin shows a stronger binding to proteins than ampicillin (II) The uptake into liver parenchyma may be a consequence of binding to liver cell components Ultrafiltration studies revealed binding of PcV to liver homogenate A slight decrease in binding of PcV was noted when the homogenate also contained BSP and DNP, which may be interpreted as competition for tissue binding sites The gel-filtration studies revealed no apparent binding of PcV to the liver cytosol protein fractions Y and Z, which are said to be of importance of biliary excretion of organic anions such as those of BSP and bilirubin (Lewi et al, 1969) However, these authors also found organic anions, e g those of probenecid and novobiocin, which did not bind to these protein fractions and the same may be valid for PcV The observed inhibition of PcV uptake with e g BSP and probenecid may be related to competition for structures of the cellular membrane of the hepatocytes Cornelius et al, (1967) have shown competition for various organic anions at isolated hepatic cell plasma membranes

No effect on the uptake of PcV with organic cations such as emepronium, methylatropine and the conjugate acid of lidocaine was obtained Emepronium and methylatropine are quaternary ammonium compounds and lidocaine is a tertiary amine This is in agreement with the findings of Schanker (1965 & 1968) He has suggested that choleophilic (prone to biliary excretion) organic cations and anions are excreted by separate systems in the liver

Quaternary ammonium compounds and tertiary amines

Biliary excretion of quaternary ammonium compounds and tertiary amines (IV)

The biliary elimination of some quaternary ammonium compounds and tertiary amines after their intravenous administration into bile fistula rats was studied The compounds were labelled with radioactive isotopes Some results are given in Table 2 and the results are discussed with regard to the importance of polarity and molecular size in biliary excretion

The obtained results showed great differences in the amounts of radio activity eliminated into the bile (0 0—54 %) The chromatographic studies revealed that in general only a small fraction of the activity eliminated into the bile was attributable to the original compounds The metabolites formed seemed to be more polar than the original substances Preliminary incubation studies with β-glucuronide and sulphatase indicated that con jugates were present in the bile This is in agreement with findings reported by other investigators Bickel & Minder (1970) have shown in rat that imipramine and desmethylimipramine are excreted into the bile mainly as

glucuronic acid conjugates of the hydroxylated drugs Keeneghan & Boyes (1972) have shown that hydroxylated lidocaine and a demethylated hydroxylated lidocaine metabolite are excreted in the bile as acid hydrolyzable conjugates

Schanker (1965) has proposed that the molecular structures may be of importance for extensive biliary excretion To be a choleophilic organic

Table 2 Biliary excretion of radioactivity after intravenous administration of some quaternary ammonium compounds and tertiary amines into rats

Compound	Molecular*/ weight	Dose mg/kg	Time of collection of bile (hrs)	% of total dose collected in bile, mean and range
Quaternary ammonium compounds				
3 acetylpyridine				
methiodide ^{14}C				
1-(3 pyridyl) ethanol	138	0 3	6	0 5 (0 4—0.5)
methiodide ^{14}C				
n cotinine iso-	177	0 2	6	0 0
methiodide ^{14}C				
cotinine	206	0 2	6	0 0
methiodide ^{14}C				
emepronium ^{14}C	283	0 2	4	28 5 (13 4—39 8)
methylatropine ^{3}H	303	0 5	4	20 7 (20 2—21 0)
N hydroxyethyl	330	1 0	4	21 2 (12 3—36 4)
prometazine ^{35}S				
piribenzil ^{3}H	355	1 0	4	28 9 (23 0—34 0)
Tertiary amines				
nicotine ^{14}C	162	0 4	6	2 7 (2 0—3 5)
lidocaine ^{14}C	234	4 2	4	33 5 (29 9—37 0)
mepivacaine ^{14}C	246	6 6	6	54 3 (51 4—56 5)
amitriptyline ^{14}C	277	0 8	6	47 2 (42 9—51 4)
imipramine ^{14}C	284	2 0	6	35 3 (34 7—35 9)
prometazine ^{35}S	284	1 0	4	18 1 (9 8—22 7)

* The molecular weights are calculated on the free onium compounds and amines

cation the molecule should have a positively charged amino group at one end of the molecule and one or more non-polar ring structures at the opposite end separated by an intermediate chain In the compounds used in this investigation showing extensive biliary excretion, the molecular properties suggested by Schanker can be found The tertiary amines used in the present investigation may also be taken as an exemple of this since the amino group is protonated to a large extent in the physiological pH-range These structural properties may be of importance not only for direct excretion as

suggested by Schanker (1965), but *these molecules may be prone to undergo* biotransformation reactions involving e g hydroxylation and conjugation leading to products which are highly polar compounds of high molecular weight With the conjugation step an anionic center in the molecular is obtained The obtained metabolite(s) may be transported both by anionic and cationic systems It is possible that the conjungation step is closely associated with excretion mechanisms

Several investigators have pointed out that molecular size may be of importance for biliary excretion (e g Sperber, 1963, Williams et al, 1965 and Millburn et al, 1967) It has been suggested that compounds with molecular weights larger than about 300 are predominantly excreted in the bile It has been shown that high molecular weight alone does not lead to extensive biliary excretion For example dieldrin, Mw 389 (Williams et al, 1965), sucrose, Mw 352 and inulin, Mw >500 (Schanker & Hogben, 1961) show minimal biliary excretion High molecular weight in combination with or as a consequence of the presence of polar groups e g anionic and/or cationic, and structural features such as amphiphatic character (Smith, 1971) seem to lead to extensive biliary excretion Several investigators e g Brauer (1959), Sperber (1959 & 1963) and Schanker & Hogben (1961) have discussed some kind of porosity (permeability) of the hepato-biliary system in relation to molecular size of various compounds and biliary elimination The results from the present study showed that only small amounts (0 0—2 7 %) of the injected doses of activity were found in the bile with *the componds with an initial molecular weight up to about 200 Compounds* with an initial molecular weight above 200 showed extensive biliary excretion (18—54 %) As pointed out before, the chromatographic studies indicated that in most cases with extensive biliary elimination, most of the activity eliminated into the bile was attributable to metabolites, presumably conjugates This could lead to the initial molecular weight increasing with *100—200* units, depending on the *conjugating agent e g sulphuric acid* — glucuronic acid As a conclusion, the importance of molecular size for biliary excretion is imperfectly understood A more definite conclusion could be drawn if primary bile was accessible for analysis

Besides the above mentioned aspects of biliary excretion it must be re membered that with the technique used the kidneys are functionally active and there is competition between excretion in *the bile and urine*

Transport studies with piribenzil (V)

To elucidate transport characteristics of the liver parenchyma for piribenzil, a cationic compound with anticholinergic properties incubation studies with liver slices were performed In this study, tritium labelled compond was used

It was shown that the uptake of piribenzil was concentration and time dependent At a medium concentration of 10 μmole/l the S/M ratio was about 4 after 60 minutes incubation To study if the uptake into liver slices was energy dependent (e g ATP dependent) incubation studies were performed with metabolic inhibitors such as IAA and DNP Also incubation in a N_2-atmosphere was performed The results clearly showed that with these incubation conditions, a statistically significant decrease in the uptake of piribenzil was noted compared with control values This effect was seen already after 5 minutes incubation and statistically significant after 10 minutes incubation and probably reflects events at perisinusoidal (and intercellular) sites of the liver parenchyma Solomon & Schanker (1963) found that the uptake of procaine amide ethobromide (PAEB), another quaternary compound, was inhibited by IAA, DNP and incubation in N_2-atmosphere These results are also in agreement with what has been found in uptake studies with the amino compound chlorguanide triazine (Gigon et al, 1969) The uptake of decamethonium into liver slices have also been shown to be energy dependent (Broen Christensen, 1970)

The uptake of bisquaternary ammonium compounds such as hexaflorenium and tubocurarine was inhibited by cardiac glycosides e g K-strophantoside, digitoxin and ouabain (Meijer et al, 1971) Inhibition of the uptake of piribenzil was only noted at very high concentrations of ouabain (1 mmole/l) (an inhibitor of Na^+, K^+ — ATP-ase) No effect on the uptake was noted when the concentration of ouabain in the medium was 100 μmole/l It is interesting to note that the biliary excretion of PAEB was inhibited by bisquaternary ammonium compounds such as tubocurarine and hexaflorenium but not influenced by cardiac glycosides (Meijer et al, 1970) These authors suggested that the transport of mono- and bisquaternary agents may differ in at least one step

In efflux studies with liver slices preloaded with ^3H piribenzil, it was found that IAA decreased the release of radioactivity from the slices This effect may suggest the presence of an energy dependent transport system direct out from the liver parenchyma However, the present study does not indicate whether such a transport system is localized to canalicular sites and or to other sites of the liver parenchyma

Other mechanisms than energy dependent uptake may also be responsible for the uptake of organic cations into the liver slices e g binding to liver cell components. Ultrafiltration studies showed extensive binding of piribenzil to liver homogenate This may be due to binding to components (e g proteins) of the cell wall as well as of intracellular components Tissue binding seems to be of varying importance for various organic cations. Piribenzil as well as chlorguanide triazine (Gigon et al, 1969) show extensive

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It was shown that the uptake of piribenzil was concentration and time dependent At a medium concentration of 10 μmole/l the S/M ratio was about 4 after 60 minutes incubation To study if the uptake into liver slices was energy dependent (e g ATP dependent) incubation studies were performed with metabolic inhibitors such as IAA and DNP Also incubation in a N_2-atmosphere was performed The results clearly showed that with these incubation conditions, a statistically significant decrease in the uptake of piribenzil was noted compared with control values This effect was seen already after 5 minutes incubation and statistically significant after 10 minutes incubation and probably reflects events at perisinusoidal (and intercellular) sites of the liver parenchyma Solomon & Schanker (1963) found that the uptake of procaine amide ethobromide (PAEB), another quaternary compound, was inhibited by IAA, DNP and incubation in N_2-atmosphere These results are also in agreement with what has been found in uptake studies with the amino compound chlorguanide triazine (Gigon et al, 1969) The uptake of decamethonium into liver slices have also been shown to be energy dependent (Broen Christensen, 1970)

The uptake of bisquaternary ammonium compounds such as hexaflorenium and tubocurarine was inhibited by cardiac glycosides e g K strophantoside, digitoxin and ouabain (Meijer et al, 1971) Inhibition of the uptake of piribenzil was only noted at very high concentrations of ouabain (1 mmole/l) (an inhibitor of Na^+ K^+ — ATP-ase) No effect on the uptake was noted when the concentration of ouabain in the medium was 100 μmole/l It is interesting to note that the biliary excretion of PAEB was inhibited by bisquaternary ammonium compounds such as tubocurarine and hexaflorenium but not influenced by cardiac glycosides (Meijer et al, 1970) These authors suggested that the transport of mono- and bisquaternary agents may differ in at least one step

In efflux studies with liver slices preloaded with ^{3}H piribenzil, it was found that IAA decreased the release of radioactivity from the slices This effect may suggest the presence of an energy dependent transport system direct out from the liver parenchyma However, the present study does not indicate whether such a transport system is localized to canalicular sites and/or to other sites of the liver parenchyma

Other mechanisms than energy dependent uptake may also be responsible for the uptake of organic cations into the liver slices e g binding to liver cell components Ultrafiltration studies showed extensive binding of piribenzil to liver homogenate This may be due to binding to components (e g proteins) of the cell wall as well as of intracellular components Tissue binding seems to be of varying importance for various organic cations Piribenzil as well as chlorguanide triazine (Gigon et al 1969) show extensive

suggested by Schanker (1965), but these molecules may be prone to undergo biotransformation reactions involving e g hydroxylation and *conjugation* leading to products which are highly polar compounds of high molecular *weight* With the conjugation step an anionic center in the molecular is obtained The obtained metabolite(s) may be transported both by *anionic* and cationic systems It is possible that the conjungation step is closely associated with excretion mechanisms

Several investigators have pointed out that molecular size may be of importance for biliary excretion (e g Sperber, 1963, Williams et al, 1965 and Millburn et al, 1967) It has been suggested that compounds with molecular weights larger than about 300 are predominantly excreted in the bile It has been shown that high molecular weight alone does not lead to extensive biliary excretion For example dieldrin, Mw 389 (Williams et al, *1965*), sucrose, *Mw 352 and inulin, Mw >500 (Schanker & Hogben, 1961)* show minimal biliary excretion High molecular weight in combination with or as a consequence of the presence of polar groups e g anionic and/or cationic, and structural features such as amphiphatic character (Smith, *1971*) seem to lead to extensive biliary excretion Several investigators e g Brauer (1959), Sperber (1959 & 1963) and Schanker & Hogben (1961) have discussed some kind of porosity (permeability) of the hepato biliary system in relation to molecular size of various compounds and biliary elimination The results from the present study showed that only small amounts (0 0—2 7 %) of the injected doses of activity were found in the bile with the componds with an initial molecular weight up to about 200 Compounds *with an initial molecular weight above 200 showed extensive biliary ex*cretion (18—54 %) As pointed out before, the chromatographic studies indicated that in most cases with extensive biliary elimination, most of the activity eliminated into the bile was attributable to metabolites, presumably conjugates This could lead to the initial molecular weight increasing with 100—200 units, depending on the conjugating agent e g sulphuric acid — glucuronic acid As a conclusion, the importance of molecular size for biliary excretion is imperfectly understood A more definite conclusion could be drawn if primary bile was accessible for analysis

Besides the above mentioned aspects of biliary excretion it must be remembered that with the technique used the kidneys are functionally active and there is competition between excretion in the bile and urine

Transport studies with piribenzil (V)

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SUMMARY

The biliary excretion of some penicillins (examples of compounds giving anions) and quaternary ammonium compounds and tertiary amines (examples of cations or compounds giving cations) have been studied in bile fistula rats after intravenous administration The biliary excretion of these compounds are discussed with regarded to their molecular properties e g molecular weight, polarity and structure To elucidate transport characteristics of the liver parenchyma for such compounds, incubation studies with rat liver slices were performed Some of the penicillins and all of the quaternary ammonium compounds and tertiary amines were labelled with radioactive isotopes

In the excretion studies with penicillins high excretion of biologically active penicillin was noted These compounds giving anions have molecular weights above 300 The amount of active drug varied with the different penicillins (11—38 %) and this variation may at least partly be attributable to a difference in inactivation (e g in the liver), which is supposed to be related to the structure of the side chain of the penicillin The total excretion in the bile (drug + metabolic(s)) may roughly be the same The dominating metabolite seems to be the penicilloic acid of the respective penicillin

In the studies with the cation-forming compounds the biliary excretion of radioactivity varied from 0 0—54 % of the given doses Chromatographic studies indicated that only a smaller fraction of the activity eliminated into the bile was attributable to the original compound in most cases in which extensive excretion of radioactivity was noted Metabolites were formed, presumably conjugates which seemed to be more polar than the original compounds The attainment of high polarity and possibly high molecular weight (>300) may be of importance for biliary excretion

In the incubation studies with rat liver slices, indications of an energy dependent uptake of piribenzil a cation, into the hepatocytes were seen, but with phenoxymethylpenicillin (PcV), an anion-forming agent, no clear indication of such a process was noted The passage of PcV into the liver slices seemed mainly to be related to the metabolic degradation of the compound in the liver cells

Binding to liver homogenate was observed with both piribenzil and PcV so uptake into the liver slices may at least partly be caused by binding to liver cell components

No competition in uptake into liver slices between an organic anion and an organic cation was observed, but structural analogues inhibited each others uptake which indicates separate transport mechanism(s) for anions and cations

binding but PAEB (Solomon & Schanker, 1963) showed almost no such binding

Passage into liver slices could also be attributable to a reduction in the free concentration of piribenzil in the cytosol by biotransformation However, no clear evidence of biotransformation of piribenzil in the liver slices was obtained

The uptake of ^{3}H-piribenzil into the liver slices was decreased by other organic cations e g emepronium, methylatropine and the conjugate acid of lidocaine Organic anions such as the conjugate bases of BSP, penicillin, phenolphthalein glucuronide did not influence the uptake

REFERENCES

Acred, P, D M Brown, D H Turner & D Wright *Pharmacology of methicillin* Brit. J Pharmacol 1961, *17*, 70—81

Ayliffe, G A J & A Davies Ampicillin levels in human bile Brit J Pharmacol *1965, 24, 189—193*

Baker, K J & S E Bradley Binding of sulfobromophthalein (BSP) sodium by plasma albumin Its roles in *hepatic BSP excretion* J Clin Invest. 1966, *45*, 281—287

Bakke, O M The significance of the enterohepatic circulation in the metabolism of hormones and drugs Nordisk Medicin 1970, *8*, 1289—1320

Barber Riley, R W The removal of bromosulphthalein from solutions by tissue slices. S Afr J med Sci 1962, 27, 25—34

Bernstein, L H, I Ben Ezzer, L Gartner & J M Arias Hepatic intracellular distribution of tritium labelled unconjugated and conjugated bilirubin in normal and Gunn rats. *J Clin Invest 1966, 45,* 1194—1201

Biagi, G L, A M Barbaro, M F Gamba & M C Guerra Partition data of penicillins *determined by means of reversed phase thinlayer chromatography* J Chromatogr 1969, *41*, 371—379

Bickel, A E & R. Minder Metabolism and biliary *excretion of the lipophilic drug* molecules, imipramine and desmethylimipramine in the rat — I Biochem Pharmacol 1970, *19*, 2425—2435

Bird, A E & A C Marshall Correlation of serum binding of penicillins with partition coefficients Biochem Pharmacol 1967, *16*, 2275—2290

Bird, A E & A C Marshall Reversed phase thin layer chromatography and partition coefficients of penicillins J Chromatogr 1971, 63, 313—319

Brauer R. W Mechanisms of bile secretion J.A M.A 1959 150, 1462—1466

Brauer, R W & R. L. Pessotti The removal of bromsulphthalein from blood plasma by the liver of the rat *J Pharmacol* Exp. Ther *1949, 97*, 358—370

Braun, W R, G M Grodsky & J V Carbone Intracellular distribution of tritiated bilirubin during hepatic uptake and excretion Amer J Physiol 1964, *207*, 1237—1241

Broen-Christensen, Chr In vitro studies on the uptake of [14]C labelled decamethonium and hexamethonium in mouse liver Acta pharmacol et toxicol 1970, *28*, 215—224

Coppi, C L Monti & R Genova Choleretics and biliary excretion of antibiotics in rats Chemotherapy 1971, *16* 345—355

Cornelius C E, J Ben Ezzer & J M Arias Binding of sulfobromophthalein sodium (BSP) *and other organic anions by isolated hepatic cell plasma* membranes in vitro Proc Soc Exp Biol Med 1967, *124* 665—667

Forker, E L Two sites of bile formation as determined by mannitol and erythritol clearance in the guinea pig J Clin Invest 1967, *46* 1189—1195

Gigon P L, P K Nayak & L S Schanker Transport and binding of chlorguanide triazine Proc Soc Exp Biol Med 1969 *132* 1103—1109

Hargreaves, T & V Price The uptake of conjugated bilirubin by rat liver Biochem Pharmacol 1966, *15* 657—667

Harrison, P M & G T Stewart Excretion of antibiotics in the bile Brit J Pharmacol 1961, *17* 420—423

Henegar, G M Silverman R J Gardner, J C Kuhrol & R W Preston Excretion of methicillin in human bile *Antimicrob Ag Chemother 1961*, 348—351

Keenaghan J B & R N Boyes The tissue distribution, metabolism and excretion of lidocaine *in rats, guinea pigs* dogs and man J Pharmacol Exp Ther 1972, *180*, 454—463

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Warrick, C K Some aspects of the radiology of the biliary system In The biliary system Ed W Taylor, Oxford, Blackwell, 1965, p 637—645

Wheeler, H O & O L. Ramos Determinants of the flow and composition of bile in the unanesthetized dog during constant infusions of sodium taurocholate J Clin Invest 1960, *39*, 161—170

Wheeler, H O Water and electrolytes in bile In Handbook of physiology Alimentary canal 1968 Sect. 6, Vol 5, Chapt, 113, p 2409—2431

Wheeler, H O, E D Ross & S E Bradley Canalicular bile production in dogs Amer J Physiol 1968, *214*, 866—874

Williams, R T, P Millburn & R L. Smith The influence of enterohepatic circulation on toxicity of drugs. Ann N Y Acad Sci 1965, *123*, 110—124

Kind, A C, H N Beaty, L F Fenester & W M M Kirkby Inactivation of pen cillins by the isolated rat liver J Lab Clin Med 1968, *71* 728—735

Kriegistein, J, W Meiler & J Staab Hydrophobic and ionic interactions of phenothia zine derivatives with bovine serum albumin Biochem Pharmacol 1972, *21*, 985—997

Lewi, A J, Z Gatmaitan & *J M Arias Two hepatic* cytoplasmic proteins fractions Y and Z, and their possible role in the hepatic uptake of bilirubin, sulfobromophtha lein and other anions J Clin Invest 1969, *48*, 2156—2167

Meijer, D K F, E S Bos & K J van der Laan Hepatic transport of mono and bisquaternary ammonium compounds European J Pharmacol 1970 *11*, 371—377

Meijer, D K F, *J W* Arends & J G Weitering The cardiac glucoside sensitive step in the hepatic transport of the bisquaternary ammonium compound, hexaflorenium European J *Pharmacol 1971, 15, 245—251*

Millburn, P, R L Smith & R T Williams Biliary excretion of *foreign compounds* —
at molecular weight, polarity and
J 1967, *105* 1275—1281

Ockner, R. K, *J* A Manning, R B Poppenhausen & W K L Ho A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium and other tissues Science, 1972, *177*, *56—58*

Schanker, L S Hepatic transport of organic cations In The biliary system Ed, *W Taylor, Oxford Blackwell, 1965,* p *469—480*

Schanker, L.S Secretion of organic compounds In Handbook of physiology Ali mentary canal *1968* Sect 6, Vol *5*, Chapt 4, p 2433—2449

Schanker, L S & C A Hogben Biliary excretion of inulin, sucrose and mannitol ana lysis of bile formation Amer J Physiol 1961, *200*, 1087—1090

Smith, R L The biliary excretion and enterohepatic circulation of drugs and other organic compounds In Progress in drug research Vol 9 Ed E Jucker Birkhauser Verlag, Basel 1966, p 299—360

Smith, R L Excretion of drugs in bile In Handbook of experimental pharmacology, 1971, Vol XXVIII/1 p 354—389

Solomon H M & L S Schanker Hepatic transport of organic cat ons active uptake of a quaternary ammonium compound, procaine amide ethobromide by rat liver slices Biochem Pharmacol 1963, *12* 621—626

Sperber, I Secretion of organic anions in the formation of urine and bile Pharmacol Rev 1959, *11*, 109—134

Sperber, I Biliary excret on and choleresis In Proc 1st int pharmacol Meet, Vol 4 Drugs and membranes 1963 p 137—143

Sperber, I Biliary secretion of organic anions and its influence on bile flow In The biliary system Ed, W Taylor, Oxford, Blackwell 1965 p 457—467

Sperber, I & G Sperber Hepatic excretion of radiocontrast agents In International encyclopedia of pharmacology and therapeutics Ed P Knoefel Pergamon Press 1971, Vol 1 p 165—235

Stewart, G T & P M Harr son Excretion and re excretion of a broadspectrum peni cillin in bile Brit J Pharmacol 1961 *17* 414—419

Vanlerenberghe J, C Bel N Turpin & R Guislain Inhibition enzymatic et conju gaison biliare de la brome sulfone phtaleine Arch internat Physiol Biochem 1970 *78*, 935—951

Walpole, A L M H Williams & D C Roberts The carcinogenic action of 4 amino diphenyl and 3 2'dimethyl-4 aminodiphenyl Brit J industr Med 1952 *9* 255—263

Warrick, C. K. Some aspects of the radiology of the biliary system. In: The biliary system. Ed. W. Taylor, Oxford, Blackwell, 1965, p. 637—645
Wheeler, H. O. & O. L. Ramos. Determinants of the flow and composition of bile in the unanesthetized dog during constant infusions of sodium taurocholate. J. Clin. Invest. 1960, *39*, 161—170
Wheeler, H. O. Water and electrolytes in bile. In: Handbook of physiology. Alimentary canal. 1968. Sect. 6, Vol. 5, Chapt. 113, p. 2409—2431
Wheeler, H. O., E. D. Ross & S. E. Bradley. Canalicular bile production in dogs. Amer. J. Physiol. 1968, *214*, 866—874
Williams, R. T., P. Millburn & R. L. Smith. The influence of enterohepatic circulation on toxicity of drugs. Ann. N. Y. Acad. Sci. 1965, *123*, 110—124